

# STUDIES ON THE OVIDUCTAL EPITHELIUM OF THE RAT AND THE RABBIT

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## STUDIES ON THE OVIDUCTAL EPITHELIUM OF THE RAT AND THE RABBIT (ABSTRACT)

The morphology of the rat and rabbit oviductal epithelium was examined at the light and electron microscopic level. The structure of the two main cell types, i.e. secretory and ciliated, was discussed in relation to the function of the epithelium. Other features of the oviductal epithelium which were examined included ciliogenesis, ciliary vacuoles, paracrystalline mitochondrial inclusions, 'transitional' epithelial cells and 'wandering' cells. The role played by estradiol in the regulation of the behaviour of the epithelial cells was discussed.

The effect that short-term exogenous estradiol had on the oviductal epithelium of the immature rat and rabbit was studied. Estrogen treatment did not alter the course of differentiation occurring in the developing oviducts of either species. Estrogen injections caused significant increases in cell and nuclear volume and in cellular protein synthesis (as indicated by increased nucleolar diameter and proliferation and dilatation of rough endoplasmic reticulum) of the oviductal epithelial cells. Changes provoked in the hypothalamico-pituitary-ovarian axis of the immature rat by the exogenous estrogen led to release of progesterone from the animals' ovaries. Eventually the endogenous progesterone caused a decrease in cell and nuclear volume and nucleolar diameter. The combined action of the two hormones resulted in the formation of intracellular microvillous vesicles in the oviductal epithelium of the immature rat. There was no evidence of progesterone secretion from the ovaries of the estrogen-treated immature rabbits.

The effect that long-term estrogen treatment had on the oviductal epithelium of the rat and rabbit was also examined. Oviductal infections which developed in the experimental rats were one consequence of the state of 'pseudo-pregnancy' which was maintained during the first two months of estrogen treatment. The combined estrogenic and progestational

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stimulation of the rat oviduct during this period led to the formation of many 'intraepithelial mucus inclusions' (intracellular microvillous vesicles). Following cessation of luteal function, prolonged estrogenic stimulation caused proliferative changes in the epithelium. Adenomyosis and stratified squamous metaplasia were also seen. Changes observed in the oviducts of estrogen-treated rabbits included hyperplasia, adenomyosis and neoplasia.

Salpingitis caused by a reo-virus was detected in three of the experimental rabbits. The exclusive infection of the ciliated cells of the epithelium was related to the affinity of reo-viruses for host-cell microtubules.

The morphology of the rat oviductal epithelium was also studied following 2-9 days in organ culture in a brief examination of this experimental technique.



STUDIES ON THE OVIDUCTAL EPITHELIUM OF THE RAT AND THE RABBIT

by

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A thesis presented for the degree of Doctor of Philosophy of  
the University of St. Andrews.

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June 1976



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#### SUPERVISOR'S CERTIFICATE

I certify that Eleanor Leonard Munro has fulfilled the conditions laid down under Ordinance General No.12 and Resolution of the University Court, 1967, No.1, and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

#### DECLARATION

I declare that the work presented in this thesis is my own and has not previously been submitted for any other degree.

#### CURRICULUM VITAE

I graduated from Penn Hills Senior High School (Pittsburgh, Pa., USA) in 1967. I attended Oberlin College (Oberlin, Ohio, USA) from 1967-1969. I continued my university education at St. Andrews University where I graduated with an honours degree in Anatomy. The work described in this thesis was carried out between October, 1972 and July, 1975.

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## SUMMARY

The morphology of the rat and rabbit oviductal epithelium was examined at the light and electron microscopic level. The structure of the two main cell types, i.e. secretory and ciliated, was discussed in relation to the function of the epithelium. The role played by estradiol in the regulation of the behaviour of the epithelial cells was also discussed.

The presence of ciliary vacuoles in the epithelium of the rat and rabbit oviducts was noted and a detailed examination of the morphology of these structures was performed. These intracellular vacuoles containing cilia were observed to be formed in some instances by the fusion of distended segments of RER, and in other cases through the coalescence of several small vesicles which, by their relationship to developing cilia, were designated as primary ciliary vesicles. It was observed that the mechanism of centriole replication occurring in association with ciliary vacuole formation differed considerably from the fibro-granular precursor pathway normally seen in ciliogenic cells of the oviductal epithelium. No explanation could be found for the development of ciliary vacuoles; nor was the eventual fate of these structures determined.

Paracrystalline inclusions found in the mitochondria of the ciliated cells of the rat oviduct were described in some detail, and discussed in relation to other types of mitochondrial inclusions cited in the literature. 'Transitional' epithelial cells, degenerating cells and 'wandering' cells in the oviductal epithelium were also described and discussed in Part I of this report.

The effect that short-term exogenous estradiol had on the oviductal epithelium of the immature rat and rabbit was examined in Part II of this study. Estrogen treatment did not alter the course of differentiation occurring in the developing oviducts of either species.

Estrogen injections caused a significant increase in cell, nuclear and nucleolar size in the oviductal epithelium of the immature rat during the early stages of hormone treatment. An increase in the amount and dilatation of RER in the epithelial cells was also observed. The decrease (to within control values) in cell, nuclear and nucleolar size observed following the fifth and sixth day of estrogen treatment was believed to be caused by endogenous progesterone released by the immature rats' ovaries as a consequence of alterations in the hypothalamico-pituitary-ovarian axis provoked by the exogenous estradiol. The development of microvillous vesicles in the nonciliated cells of the oviduct following six days of exogenous estrogen was also believed to be the result of the combination of progestational and estrogenic stimulation.

Estrogen treatment also provoked increases in the cell and nuclear volumes of the oviductal epithelium of the immature rabbit.

However only two of the estrogen-treated rabbits showed increases in nucleolar size and in only one of these animals was there a visible stimulation of protein synthesis in the epithelial cells. The results of the study using immature rabbits were discussed in relation to the possibility that not all of the experimental animals had received the same hormone treatment.

In Part III of this study, the effect that prolonged release of estrogen from a subcutaneous pellet had on the oviductal epithelium of the rat and rabbit was examined. Initially the estrogen treatment provoked a state of 'pseudopregnancy' in the rat, and the increase in endogenous progesterone output during the first two months of hormone treatment had several effects.

Changes elicited by progesterone in the cervix and uterus were believed to be responsible for the influx of bacteria and the establishment of reproductive tract infections, which eventually spread to the oviducts of the estrogen-treated rats. The combination of estrogenic and progestational stimulation of the rat oviducts was held responsible for the development of many epithelial mucus inclusions. Electron microscopic examination of these Alcian Blue positive inclusions revealed them to be intracellular microvillous vesicles. These vesicles appeared to develop in a manner analogous to that observed during ciliary vacuole formation.

Endogenous progesterone secretion also appeared to prevent the formation of proliferative nodules in the rat oviductal epithelium. After the cessation of luteal function approximately 2-3 months following pellet implantation, foci of epithelial 'proliferation' appeared in the rat oviduct. Adenomyosis and stratified squamous metaplasia were also observed in the oviducts of estrogen-treated rats. In addition, proliferative and metaplastic changes, and a few cases of endometriosis, were observed in the oviductal serosal mesothelium of estrogen-treated rats.

Long-term estrogen treatment led to an accumulation of secretory material in the epithelial cells of the rabbit oviduct,



which, in the absence of progestational stimulation, was not released. The retention of the secretory product appeared to have a deleterious effect on the cells; in some areas breakdown of the basal cell membranes led to the leakage of secretory material through gaps in the basal lamina into the lamina propria.

Prolonged estrogenic stimulation also led to a diffuse hyperplasia of the rabbit oviductal epithelium. Adenomyosis was observed in the oviduct of an estrogen-treated rabbit, as was a lesion which appeared to be a carcinoma of low-grade malignancy.

A viral-induced salpingitis was observed in three of the five estrogen-treated rabbits. The morphology of the viral particles detected in the epithelium led to the identification of the virus as a member of the reovirus class. The fact that virus particles were only found in the ciliated cells of the epithelium was discussed in relation to observations cited in the literature related to the association of reoviruses with host-cell microtubules.

The role of estrogen in the induction of metaplasia in the reproductive epithelia was discussed. The observation that long-term estrogenic stimulation can lead to proliferative changes in metaplastic epithelium suggested a hypothetical mechanism for hormonally-induced neoplasia.

In the Appendix to this report the results of several pilot organ culture experiments were presented. The morphology of the rat oviductal epithelium was described following 2-9 days in culture. Several ultrastructural observations suggesting the breakdown in the homeostatic relationships between the tissues of the oviduct were recorded and discussed in relation to the interpretation of results from organ culture studies.

## ADDENDUM: SOURCES OF EXPERIMENTAL ERROR

Part II of this thesis contains a report of the effects of short-term administration of estradiol on the oviductal epithelium of the immature rat and rabbit. In the discussion of this part of the thesis, conclusions pertaining to the effects of estrogen treatment on cell, nuclear and nucleolar size are presented. As a result of certain faults in the design of the experiments, these conclusions were based on insufficient data. Evaluation of the results of these experiments must be made with an appreciation of the following sources of experimental error.

### Sample Numbers and Controls

~~An~~ insufficient number of rats and rabbits was used both with respect to the control and experimental animals.

In the rat experiment only one animal per day was sacrificed for each of the six days following the initiation of the estrogen treatment. Basing conclusions concerning patterns of change during estrogen treatment when only one animal is sacrificed each day following initiation of treatment does not take into account the possibility of individual variation in the pre-experimental parameters nor does it allow for individual variation in response of the oviductal epithelium to a given hormonal stimulus.

Control animals in the rat experiment, were examined at one and at six days only. Good experimental design would require that a control animal be provided each day. It could be suggested, for instance, that the injection of the control material (corn oil) for two days could have a different effect on the oviductal epithelium from the injection of the material each day for six days.

The same kind of criticism can also be made of the rabbit experiments. Although two rabbits were sacrificed after three days of estrogen treatment, and two following four days of treatment, the results from these animals were shown separately because the animals came from different litters (see page 42).

It is also pointed out that good experimental design in the rabbit experiment requires that each group of four littermates should include one intact control and one ovariectomised control animal and not (as in this report) two control animals in each of the first two litters and no control from the third litter.

#### Dosage of estradiol

As regards dosage it is now appreciated that the effects of the same dose of estradiol cannot be compared in rat and rabbit because of the gross difference in body weight in the two species. Furthermore it is also appreciated that within each species the weight of individual animals should have been measured so that individual doses could have been related to units of body weight.

Noyes, Adams and Walton (1959) using the same dose of estradiol in rabbits as in the present experiments, ie 1  $\mu$ g, found that this amount was sufficient to maintain the uterine weight at the level found in the estrus animals weighing approximately 3.5kg. It was therefore believed that 1  $\mu$ g of estradiol would be non-toxic, but sufficiently above the physiological level, to induce a response in the reproductive tract in the immature rabbit of weight 1-2 kg.

In the present study however the daily administration of 1  $\mu$ g estradiol benzoate to intact immature rabbits elicited an increase in uterine weight in only three out of eight rabbits. This suggests that for some of the rabbits the 1  $\mu$ g dosage was within physiological limits.

It is also noted that greater reliance could have been placed on the effects of the estrogen treatment on uterine weight if the weights had been recorded as gms uterine weight/kg body weight.

The dose of estradiol given to the immature rabbits is well above the physiological level for the immature rats used in the present study. One microgram of estradiol given to rats weighing 50-75 gms is equivalent to a dosage of 14-20  $\mu\text{g/kg/day}$ , whereas the researches of McPherson et al. (1974) showed that subcutaneous injections of estradiol benzoate in doses greater than 0.3 - 0.4  $\mu\text{g/kg/day}$  caused an increase in uterine weight above the level of intact controls when administered for five days to ovariectomised twenty one day old rats. So it must be accepted that the dose used in the rats in the present investigation is pharmacological rather than physiological.

Ultimately therefore an attempt was being made to compare the effects that a near physiological (i.e.  $< 1 \mu\text{g estradiol/kg BW}$ ) dose of estrogen had on the oviductal epithelium of the immature rabbit to the effects that a pharmacological (i.e. 14-20  $\mu\text{g estradiol/kg BW}$ ) dose of estrogen had on the same tissue in the immature rat. In addition, the results of both of these experiments were compared to those of Verhage and his colleagues (Verhage et al., 1973b). In these experiments the immature Beagles were receiving 150  $\mu\text{g estradiol-}\beta\text{ valerate/kg BW/day}$ .

The validity of the results presented in Part II of this thesis has been lessened as a consequence of these errors in experimental design. In addition, in the discussion of this part of the thesis a hypothesis was proposed to account for the results of the rat experiment.

This hypothesis was based on previously published experimental data. Unfortunately no evidence of a back-up nature was produced in the present experiment to substantiate the hypothesis. Histological examination of the ovaries of the estrogen treated rats and/or blood hormone assays would have considerably strengthened the case presented here.

## INTRODUCTION

### History of the Science of Reproductive Endocrinology

On the first of June 1889 the French physician Brown-Séquard announced to a congregation of the Societe de Biologie de Paris that he had felt an amazing revitalisation following a self-administered injection of an extract of dog testis. He was seventy-two years old at the time and his overwhelming enthusiasm propelled the medical fraternity into further investigations. His startling 'fountain-of-youth' proclamation has gone down in history as heralding the modern science of endocrinology.

One of the first problems to be examined in the field of reproductive endocrinology was the regulation of the period of sexual receptivity, commonly known as 'heat', displayed by many female mammals. These changes in the behaviour of the female towards the male which culminate in mating became known as the estrus period of the reproductive cycle. In 1896 Knauer was able to re-establish the sexual cycles of ovariectomized animals with ovarian transplants, and therefore concluded that the gonads must exert a strong influence in the regulation of the estrus phenomenon. Further investigations by Marshall and Jolly in 1906 and Adler in 1911 demonstrated that estrus behaviour could be induced in the ovariectomized animal using only an ovarian extract.

At the same time anatomists were beginning to publish accounts of the changes observed in the reproductive organs in association with the behavioural aspects of the reproductive cycle. As the guinea-pig does not exhibit obvious sexual swellings during estrus, or clear cut behavioural displays apart from a willingness to copulate,

Stockhard and Papanicolaou (1917) were particularly anxious to find some morphological sign that would indicate that ovulation had occurred. Their observations on uterine and vaginal histology led to the development of the vaginal smear technique. They divided the nonfertile reproductive cycle (i.e. the estrus cycle) into four phases which were defined on the basis of changes in vaginal cytology, using the terms already accorded to the stages of the reproductive cycle (i.e. proestrus, estrus, metestrus and diestrus). They observed that ovulation in the guinea-pig occurred shortly after the appearance of the estrus vaginal smear, which consists mainly of cornified epithelial cells. The adaptation of the vaginal smear dating technique to this and other species displaying regular estrus cycles proved to be a very valuable tool in further research.

The studies of the estrus cycle and related phenomena of the guinea-pig, rat and mouse by Stockhard and Papanicolaou, Long and Evans, and Allen respectively, laid the foundations for later work which would use morphological criteria as indicators of hormonal status. These researchers observed variations in ovarian morphology which could be correlated with the changes occurring in the uterus and the vagina during the estrus cycle. As mentioned previously, the ovary had already been implicated in the control of estrus behaviour. Allen's observations led him to suspect that the ovarian follicles were producing the growth stimulus responsible for the estrus changes in the reproductive tract. His collaboration in 1923 with Doisy led to the discovery that administration of an extract of ovarian follicular fluid to ovariectomized mice resulted in the development of a cornified vaginal smear characteristic of the estrus state. (Note: Early research workers often quantified estrogenic

hormones in terms of Rat Units (RU). As defined by Allen and Doisy (1924), a Rat Unit is the minimal quantity which will induce full estrus growth in the genital tract of an ovariectomized adult rat forty-eight hours after the first of three lcc injections given at intervals of 4-6 hours.)

Using the cornified vaginal smear as a test for 'folliculoid' substances, highly active preparations could be obtained, and in 1936 MacCorquodale, Thayer and Doisy isolated estradiol-17 $\beta$  in a crystalline form from sows' ovaries. Prior to this, two metabolites of estradiol, which also possessed 'estrogenic' activity, estriol and estrone, had been identified in urine.

During the same decade workers were establishing the importance of the corpus luteum in maintaining pregnancy and in 1934 Allen and Wintersteiner prepared crystalline progesterone from the corpora lutea of sows' ovaries. With the isolation and identification of the ovarian hormones the emphasis of further study was firmly shifted from the 'estrus' cycle to the 'ovarian' cycle, and the manner in which the secretions of the ovary could regulate the mammalian reproductive cycle (see Parkes and Deanesly, 1966; Selye, 1947; Young, 1961; and Zuckerman, 1962 for historical references).

#### The Ovarian Cycle and the Hypothalamico-Pituitary-Ovarian Axis

Research carried out in the field of reproductive endocrinology subsequent to these early pioneering studies has been complicated by the variation encountered in the estrus and ovarian cycles of the mammalian species under study. For instance, the rat and the rabbit, which are two of the most commonly used laboratory animals and which were the species examined in the present study, differ fundamentally



in their ovarian cycles. The female rat isolated from the male undergoes a regular ovulatory cycle and displays behavioural estrus every 4-5 days. The rabbit, on the other hand, is a reflex ovulator, and the isolated laboratory doe remains in a state of perpetual estrus until mating is allowed to occur. This results in artificially long periods of estrogen domination. These laboratory animals also differ from their wild counterparts. The breeding activity of rats and rabbits in their natural environments is limited by intervals of ovarian quiescence or anestrus. In addition, the majority of estrus cycles during the breeding season of the wild rat would be interrupted by mating and pregnancy, and likewise the amount of time spent by the rabbit doe in estrus prior to mating would be greatly reduced.

Nevertheless the vast amount of research conducted in this field using a wide variety of mammalian species has led to a much clearer picture of the mechanisms involved in the regulation of mammalian reproduction. In his review, Everett (1961) points out that it is not just the ovary, but rather the interrelationships of the ovary, anterior pituitary and hypothalamus that govern reproduction in the female. The ovarian secretions provoke changes in other tissues, and these changes can be indicative of the prevailing hormonal milieu.

The hypothalamus controls the secretion of hormones from the anterior pituitary through various stimulating and inhibiting factors that it releases into the hypophyseal-portal blood system. These vessels carry blood from capillary beds in the hypothalamus to capillary beds in the anterior pituitary. Some of the hypothalamic factors stimulate, while others inhibit the gonadotrophic hormone-secreting cells of the anterior pituitary. The gonadotrophic hormones secreted by the anterior pituitary subsequently act on the

gonad in such a way as to regulate its functioning in oogenesis and in hormone secretion. The basic pathways involved in the control of ovarian function in mammals are illustrated in Text-Figure I.

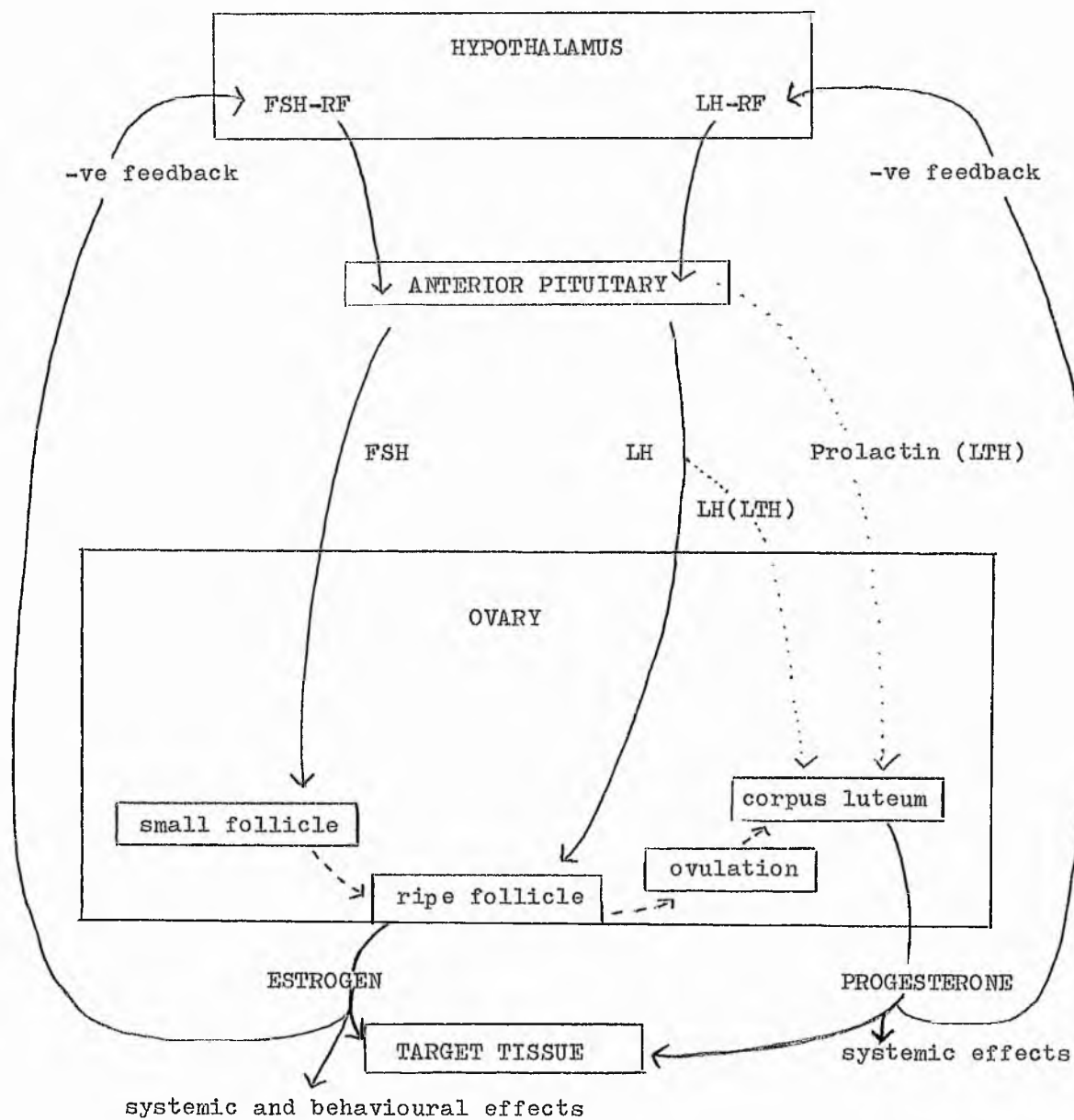
A hypothalamic releasing factor (FSH-RF) stimulates anterior pituitary cells to secrete follicle stimulating hormone (FSH). This gonadotrophin provokes small ovarian follicles to develop into mature Graafian follicles. The theca interna cells of the developing follicles are also stimulated by FSH (either directly or indirectly) to secrete estradiol. Another hypothalamic factor (LH-RF) governs the secretion of luteinizing hormone (LH) from the anterior pituitary. Luteinizing hormone acts in a synergistic manner with FSH to stimulate follicle growth and estrogen secretion, but a sudden transient increase in LH secretion causes the Graafian follicle to rupture and ovulation occurs. Following ovulation the collapsed ovarian follicle is converted to a corpus luteum whose cells are stimulated by gonadotrophins to secrete progestational hormones.

In the majority of species it seems that the formation, maintenance and hormonal secretion of the corpus luteum are controlled by LH. However in some species an additional anterior pituitary hormone, luteotrophic hormone (LTH), is responsible for the maintenance of the corpus luteum. In the rat and mouse this hormone has been identified as prolactin, and its secretion from the anterior pituitary is governed by a hypothalamic inhibiting factor (PIF).

It is the hormonal secretions of the ovary that regulate the development and functioning of the reproductive tract tissues. These hormones also indirectly influence the rate of their own secretion by the ovary via negative and positive feedback loops to the hypothalamus and the anterior pituitary. However detailing the complex-

Text-Figure I: The endocrine pathways involved in the control of ovarian regulation of target tissues.

(Slightly modified diagram from Perry, 1971)



ities of the hypothalamico-pituitary-ovarian axis is beyond the intention of this introduction. For the purpose of understanding the regulation of reproductive tract tissues by ovarian hormones it is sufficient to consider that the event of ovulation theoretically divides the ovarian cycle into two phases, one dominated by estrogen and the other by progesterone. It should be noted however that there is considerable overlap in the secretion of these hormones by the ovary, and that the interaction of the two hormones with respect to the responses elicited in their target tissues can be both synergistic and antagonistic.

The recent development of sophisticated competitive-protein-binding assays and radioimmunoassays has made it possible to detect and measure even small quantities of hormones circulating in the blood. With these methods the hormonal output from both the ovaries and the anterior pituitary can be monitored. A brief review at this point of the current knowledge of the physiological fluctuations in blood hormone levels that occur in the rat and rabbit normally will lay the groundwork for a later understanding of the manner in which exogenous hormones upset the regulation of ovarian output by the hypothalamus and pituitary.

The estrus cycle of the unmated laboratory rat can be divided on the basis of vaginal cytology into four stages, each lasting approximately one day: proestrus, estrus, metestrus and diestrus. Rats having a five-day cycle display diestrus smears for two days (Naftolin, Brown-Grant and Corker, 1972).

Release of FSH from the pituitary of the rat reaches a peak early on the day of estrus, prior to ovulation. This presumably initiates the growth of new follicles for the next cycle. These

follicles begin to secrete estradiol early on the day of diestrus and the level of plasma estradiol continues to rise until noon on the day of proestrus. About 3-5 hours following this peak in estradiol secretion there is a very sharp rise in the level of LH in the plasma. This increase in LH secretion is transitory and when the level of this hormone in the blood reaches a low base line level on the morning of estrus, it remains at this level until the next proestrus (Butcher, Collins and Fugo, 1974).

Ovulation in the rat occurs approximately 10-12 hours after the LH peak (Everett, 1961). It was originally believed that the corpora lutea of the unmated rat were nonfunctional. However plasma steroid levels indicate that there is active secretion of progesterone by the corpora lutea starting on the morning of metestrus and continuing for approximately twenty-four hours. Extension of luteal function by one day is reported for those rats having a five-day cycle. Another transitory peak of progesterone secretion occurs on the afternoon of proestrus. This is probably due to stimulation of ovarian interstitial tissue by either the LH peak (Armstrong, 1968a&b) or by prolactin, which also reaches a peak during the afternoon of proestrus. Prolactin is known to be the luteotrophic hormone (LTH) in the rat, and plasma levels of this hormone reach a second peak on the afternoon of estrus (Butcher, Collins and Fugo, 1974).

Ovarian steroid output has also been examined in the rabbit during estrus (Eaton and Hilliard, 1971) and following ovulation induced by coitus (Hilliard and Eaton, 1971). A correlation was found between the amount of estrogen being secreted by the ovary and the number and size of follicles present in that ovary. These authors also confirmed that the main source of progesterone in the

rabbit is the corpus luteum and that the large amount of interstitial tissue contributes primarily 20 $\alpha$ -hydroxypregn-4-en-3-one. The latter progestin is much less active than progesterone and although secreted in larger quantities cannot maintain pregnancy in the absence of a corpus luteum (Perry, 1971).

Copulation stimulates a LH surge in the rabbit which is followed 10-12 hours later by ovulation. Estradiol has been established as the primary luteotrophin in this species (Keyes and Nalbandor, 1967). Although the output of all three ovarian steroids is at its lowest level at ovulation, there is evidence that during the transport of the ovum through the oviduct the level of estradiol is beginning to rise again as a new crop of follicles develop. This estrogen stimulates the developing corpora lutea to secrete progesterone, and therefore the output of this hormone also increases during oviductal transport (Hilliard and Eaton, 1971).

#### Estradiol and 'Target' Tissues

The advent in the late 1950's of tritium-labeled steroids of high specific activity made it possible to administer and trace physiological doses of estradiol. The concept of 'target' tissues evolved when it was observed that certain tissues were capable of selectively retaining radioactive estradiol. The ability of these tissues to take up estradiol from the blood and retain it against a large concentration gradient indicated the presence of molecules in these tissues capable of binding the hormone. These molecules were designated as estradiol 'receptors'. Specific receptor molecules for progesterone have also been found (Jensen and DeSombre, 1972; Kumra et al., 1974).

The selective uptake of estradiol, indicative of the presence of receptors, has been demonstrated in many reproductive tissues, including the oviducts of women (Flickinger, Muechler and Mikhail, 1974; Roy et al., 1972a; Taylor, Brush and King, 1969), rats (Sen and Talwar, 1973; Stumpf, 1969), rabbits (Kim, Coates and Flickinger, 1967; Martin, McGuire and Pauerstein, 1970; Muechler, Flickinger and Mikhail, 1974; Roy et al., 1972b) and chickens (Cox, Catlin and Carey, 1971; Harrison and Toft, 1975). Using autoradiography of freeze-dried unfixed unembedded sections (to prevent diffusion artifact), Stumpf (1969) found that the radioactive estradiol was concentrated in the nuclei of the oviductal epithelial cells, although there was some cytoplasmic labeling. This is in keeping with the recent theories concerning the molecular mechanisms of estrogen action.

#### Molecular Mechanisms of Estrogen Action

The experimental work performed by biochemists during their search for the molecular mechanisms involved in estrogen action has focused on two estrogen-responsive target tissues, i.e. the immature or ovariectomized-adult rat uterus and the chick oviduct. Apart from minor discrepancies in results that probably arise from differences in experimental techniques, investigators have come to certain conclusions concerning estrogen action in these tissues which are quite likely to be applicable to most other estrogen-responsive reproductive organs.

The identification of specific estradiol-binding molecules in the target tissues as mentioned above, was one of the first steps towards the understanding of how estradiol acts within the cell.

Characterisation of the receptor molecules revealed them to be composed primarily of protein and to exist in an 8S form within the cytoplasm. Once estradiol enters the cell, association with the protein receptor results in a 4S receptor-steroid complex. This association is followed by the translocation of the complex to the nucleus. The transfer is temperature dependent and also involves the 'transformation' of the receptor binding unit from a 4S form to a 5S form. Once in the nucleus, the complex binds to a specific acceptor site and somehow causes the acceleration of certain nuclear and cytoplasmic biosynthetic events. The nuclear and cytoplasmic events that follow the association of the steroid-receptor complex with the cell nucleus will henceforth be referred to as the 'specific response' to estrogen (see Baulieu, 1971; Jensen and DeSombre, 1972, 1973; Mueller et al., 1972; and O'Malley and Means, 1974 for reviews concerning estradiol receptors).

Synthesis of small amounts of RNA and protein is believed to be one of the earliest features of the 'specific response'. Newly synthesized RNA and protein can be detected within one hour of estrogen administration. The RNA appears to be a new species of messenger RNA, which first appears fifteen minutes after hormone administration. This mRNA provides the template for the synthesis of a small amount of protein, known as 'induced protein' in the rat uterine system, and which appears forty minutes after hormonal stimulation (Katzenellenbogen and Williams, 1974). The function of 'induced protein' is not at present known, however Jensen and DeSombre (1972) have suggested that it might be necessary for the DNA-dependent RNA polymerase activity which follows (see below). Spelsberg et al.



(1973) have detected a similarly early synthesis of protein in the chick oviduct system which they have tentatively identified as an acidic chromatin protein. They suggested that the activity of this protein might result in genomic 'relief' from the DNA-template restricting properties of the histones.

Following these early events in the 'specific response' there is a concurrent increase in RNA synthesis and DNA-dependent RNA polymerase activity. The increase in RNA synthesis which occurs within 1-2 hours of the administration of estrogen is well documented, and the bulk of the newly synthesized RNA appears to be ribosomal RNA. That this increase is dependent on the preceding synthesis of small amounts of protein is indicated by the fact that puromycin or cyclohexamide (protein synthesis inhibitors) will prevent the estrogen-induced rise in RNA synthesis. And finally, 2-4 hours after estrogen administration there is a large increase in cytoplasmic protein synthesis, which also will not occur if any of the foregoing nuclear events are inhibited (see Hamilton, 1971; Jensen and DeSombre, 1972, 1973; Mueller et al., 1972; O'Malley and Means, 1972, 1974; and Segal and Scher, 1967 for reviews).

#### The Hormonal Regulation of Protein Synthesis

A large number of reactions are stimulated in the rat uterus in response to estrogen administration (Segal and Scher, 1967). The increase in uterine RNA and protein synthesis described above has been attributed to the action of the cytosol-nuclear receptor system found in the epithelial, stromal and muscle cells of the uterus. However some of the early responses of the uterus to estrogen,

e.g. water imbibition, increased vascular permeability and histamine release, appear to be the results of the hormone's interaction with a second receptor system (Tchernitchin, Tchernitchin and Galand, 1975). This system is found in the uterine eosinophils and, although functioning independently of the cytosol-nuclear receptor system, can affect it in certain ways (e.g. by influencing the accumulation of amino acids and ribonucleic acid precursors within the uterine cells).

In addition to the difficulties encountered in the interpretation of estrogen action due to the interaction of the two receptor systems, it has not been possible to isolate and identify any one protein species that is under estrogenic control in the rat uterus (Segal and Scher, 1967). The chick oviduct however is particularly suited for the study of hormonal regulation of protein synthesis. The egg-white proteins synthesized by the oviductal cells are produced in large amounts and can readily be detected by chemical means.

Primary stimulation of the chick oviduct by estrogen for approximately ten days causes the immature epithelium to differentiate into tubular gland, goblet and ciliated cells. A subsequent withdrawal of estrogenic stimulation causes a decrease in the size of the oviduct, protein concentration and protein synthesis. However for several weeks at least, the epithelial cells do not de-differentiate and therefore the results of a secondary hormonal stimulation of the chick oviduct will mimic the actions of the hormone in the adult hen.

Ovalbumin is synthesized by the tubular gland cells and secondary stimulation of the oviduct by estrogen results in an increase in synthesis of this protein that can first be detected three hours following hormone administration (Palmiter, Christensen and Schimke,

1970). This is preceded by an increase in the number of mRNA sequences coding for ovalbumin. The ovalbumin mRNA molecules first appear in the nuclear fraction of oviductal homogenates up to two hours after treatment, and 3-6 hours later they appear in the cytoplasmic polysomal fraction (Cox, Haines and Enitage, 1974).

Other studies have revealed that not all of the chick egg-white proteins have the same hormonal control mechanisms. Although estrogen can stimulate the goblet cells to differentiate, avidin (the egg-white protein synthesized by the goblet cells) synthesis will only occur in these cells following the administration of progesterone (O'Malley et al., 1974). Within the tubular gland cells, the synthesis of ovalbumin and lysozyme is predominantly regulated by estrogen, whereas conalbumin and ovomucoid are only maximally produced when a combination of estrogen and progesterone is administered. Palmiter and Smith (1973) found that the primary effect of the two steroids was exerted at some stage in mRNA synthesis. This led them to postulate the existence of multiple regulatory sites for the genomes coding for these two proteins, some of which could be activated by estrogen and others by progesterone. Thus, although allowing a certain amount of synthesis to be stimulated by either steroid, maximal protein production would require the action of both hormones.

A considerable amount of work needs to be done to confirm the existing evidence and to fill in the gaps in the present knowledge. For instance, there is still not conclusive evidence that the steroid hormones act by 'unmasking' the genome, and thus stimulating the transcription of specific mRNA sequences. Nor is it clear whether it is the steroid molecule itself or the 'transformed' protein

receptor which initiates the nuclear events. Although the details of the molecular mechanisms of estrogen action have yet to be elucidated, some correlations have been found between changes in cellular ultrastructure following estrogen administration and those changes detectable by biochemical means.

#### Morphological Changes following Estrogenic Stimulation

The earliest changes to be reported in the morphology of target cells have occurred in the nucleolus approximately five hours after the administration of estrogen to ovariectomized animals. Pollard (1970) found an increase in nucleolar ribonucleoprotein in sections of the vaginal epithelium of mice which had been treated with EDTA (a procedure which selectively bleaches the chromatin in ultrathin sections, thus accentuating ribonucleoprotein). At approximately the same time (i.e. 5-6 hours) following estrogen administration an increase in nucleolar size has been observed in the luminal and glandular epithelial cells of the rat uterus (Tachi, Tachi and Linder, 1972; Williams and Rodgers, 1972) and the uterine smooth muscle cells of the rat (Laguens, 1964). Approximately 20-24 hours after estrogen administration, increases in cytoplasmic volume have been observed in the luminal epithelial cells of the rat and mouse uteri (Nilsson, 1958a; Williams and Rodgers, 1972), and in rat uterine smooth muscle cells and fibroblasts (Ross and Klebanoff, 1967).

The most striking changes, however, have occurred in the cellular apparatus for protein synthesis, i.e. the ribosomes and rough endoplasmic reticulum. Approximately six hours after a subcutaneous injection of estrogen, Bo, Odor and Rothrock (1968) detected an abundance of ribosomes in the vicinity of the outer nuclear membrane

of the uterine smooth muscle cell of ovariectomized rats. In the following 6-96 hours they observed a gradual increase in the amount and dilatation of the cytoplasmic rough endoplasmic reticulum. Similar changes in oviductal epithelial cells of the chicken (Palmiter Christensen and Schimke, 1970), endometrial stroma cells of sheep (Brinsfield and Hawk, 1974) and rats (Tachi, Tachi and Linder, 1974) and uterine fibroblasts (Ross and Klebanoff, 1967) have been observed following estrogen administration.

Up to this point there has been only one report of the ultrastructure of a target tissue during the first few hours following estrogen administration. Pollard (1970) found no changes in the nucleolar ribonucleoprotein of vaginal cells thirty minutes after hormone application. This is consistent with the biochemical evidence which reports that the rise in nuclear ribosomal RNA synthesis occurs 1-2 hours after estrogenic stimulation. Therefore it is not at present known whether the biochemically detectable changes in RNA and protein synthesis which occur during the first hour following estrogen administration have any reflection in ultrastructural morphology.

#### The Maintenance of the Estrus Oviductal Epithelium

Many authors have reported changes in oviductal epithelial cells that can be correlated with the phases of the estrus or menstrual cycles (Abdalla, 1968; Allen, 1922; Brenner, 1969a; Clyman, 1966; Fredricsson and Björkman, 1962; Martinek, Kraus and Jirsová, 1967; McDaniel, Scalzi and Black, 1968; Overbeck, 1969; Patek, Nilsson and Johannisson, 1972; Rasweiler, 1972; Reinius, 1970; and Verhage et al., 1973a). In general, it is in the oviducts of the species with the longest cycles that the most profound changes occur. However

regardless of the length of the cycle, it has been found in most species that estrogen is responsible for the comparative hypertrophy of the oviductal cells that is observed during estrus. Injections of estrogen given to ovariectomized animals will restore the atrophied reproductive tract to this condition (Allen, 1938; Fredricsson, 1959 and McDaniel, Scalzi and Black, 1968). In those instances where ovariectomy has resulted in a de-differentiation of the epithelial cells, injection of estrogen by itself is capable of promoting reciliation and resumption of secretory product formation (Brenner, 1967; Nyak and Zimmerman, 1971; Verhage and Brenner, 1975).

Estrogen is therefore clearly involved in the maintenance of the specific cellular processes that characterize the differentiated oviductal epithelial cells. However, the degree to which estrogen is involved in the embryological differentiation of the oviduct is less clear.

#### Development of the Müllerian Duct

The oviduct is derived from the cranial region of the Müllerian (or paramesonephric) duct system. The Müllerian ducts develop from invaginations of the coelomic epithelium covering the mesoderm of the intermediate cell masses. Medial to the developing Müllerian ducts are the Wolffian (or mesonephric) ducts. Outpouchings of the Wolffian ducts give rise to the ureters in both sexes, and the ducts persist in the male as the canals of the epididymis, ductus deferens and ejaculatory ducts. However during the early development of the urogenital organs the two duct systems develop together in both sexes in an identical, ambisexual manner.

The caudal ends of the Müllerian ducts, passing ventrally to the Wolffian ducts, meet in the midline and the apposed caudal segments of the two ducts fuse. Together with the laterally applied Wolffian ducts they reach the dorsal wall of the urogenital sinus. At the end of the ambisexual period of development, proliferation of the epithelium of the urogenital sinus in the female contributes to the vaginal anlage, and the cranial extremities of the Müllerian ducts become the oviducts while the fused caudal segments develop into the uterus.

The primitive Müllerian epithelium contributes to a varying degree to vaginal formation in different species. For instance, in the rabbit, vagina cells derived from Müllerian epithelium predominate, whereas this only holds true for one-third to one-half of the epithelial cells of the rat and mouse vaginas. Although the Müllerian ducts are involved in the embryological formation of the human vagina, no Müllerian epithelium persists in the adult organ, with the vaginal cells in the adult female being derived wholly from the urogenital sinus and the Wolffian ducts (see Forsberg, 1973).

Apart from their contribution to vaginal formation, the Wolffian ducts atrophy during the sexual period of embryological development in females. In humans, remnants of these ducts can be found in the hilum of the ovary, the adjacent oviductal mesentery, and in the wall of the cervix and vagina (Gartner's duct).

#### Hormonal Regulation of Reproductive Tract Development

Cattle breeders frequently encounter twin births in which one of the twins, known as a freemartin, possesses certain congenital abnormalities. A theory proposed to explain the freemartin condition led

to the idea that fetal differentiation of the genital organs was under hormonal control.

A freemartin is a sexually abnormal calf born as a twin with a normal male calf. Genotypically female, the external appearance of the freemartin is usually that of a normal female calf. Internally however, elements of both male and female reproductive tracts are commonly present, and the gonads resemble either ovotestes or abnormal testes. A constant feature associated with these births is the presence of a union between the placentas of the twins with anastomoses occurring among the chorionic vessels.

Lillie proposed in 1917 (see Burns, 1961 and Wells, 1962) that the abnormal development of the female twin was caused by a hormone produced by the gonads of the male twin which reached the female via the placental anastomoses. Further analysis of this problem has confirmed Lillie's supposition. The results of these later experiments consistently indicated that the production of a specific factor which included androgens by the fetal testis during intrauterine life was essential for the subsequent development in the male of the Wolffian duct system with concomitant regression of the Müllerian ducts.

It has long been believed that the prenatal development of the female reproductive tract beyond the ambisexual stage is hormone independent. Indeed the results of several different experiments seem to support this belief. One of the most convincing pieces of evidence is that the development of the Müllerian duct system into a female type of reproductive tract occurs in the same manner in gonadectomized fetuses of both sexes as it does in normal females (see Burns, 1961).



Evidence from in vitro studies has also been used to support the notion that Müllerian duct development is hormone independent. The Müllerian ducts of chickens removed during the ambisexual phase of development are found to persist in culture whatever the sex of the donor embryo. This is also true of those ducts explanted from female embryos after the onset of sex differentiation. Those taken from male embryos at this time however, regress completely in culture (see Wolff, 1962). The results of the culture of Müllerian ducts from fetal rats and guinea-pigs are similar (see Price, Zaaier and Ortiz, 1969). However Price and her colleagues found that the differentiation, as indicated by coiling and regional demarcation, of the oviduct part of the guinea-pig Müllerian duct that occurred in culture reached a more advanced state in those explants removed at 29-30 days of gestation as compared to those removed during the ambisexual period (i.e. 26-27 days of gestation). This could imply that a stimulating agent had affected the oviduct following the termination of ambisexual development but prior to the time of explantation.

Certainly there is firm evidence that the fetal oviduct is capable of responding to estrogens. Injections of estrogens into both mammalian and bird female embryos causes accelerated development of the Müllerian duct derivatives and with high dosages extreme hypertrophy of the oviducts and uteri can be induced (see Burns, 1961). Addition of estrogen to the medium of fetal oviduct cultures has similar results (Price, Zaaier and Ortiz, 1969).

Until quite recently no evidence could be found for the production of estrogen by the fetal ovary at the time that sexual (c.f. ambisexual) differentiation is occurring. However secretion of estrogen

from the ovary of the six-day embryonic chick has now been detected (see Teng and Teng, 1975b). At this age chick embryos of both sexes still possess bilateral Müllerian ducts. Both ducts begin to involute on day eight in male chick embryos and in female embryos, the right Müllerian duct begins to regress on day nine (Teng and Teng, 1975a).

Cytoplasmic estrogen receptors have also been detected in the cells of the Müllerian duct of female chickens as early as the eighth embryonic day (Teng and Teng, 1975b). However although growth of the Müllerian duct, in terms of increases in length and wet weight, occurs from the eighth embryonic day to four days after hatching (*Ibid.*) cytodifferentiation of the oviductal epithelium does not occur in this species until 3-4 months later (Palmiter and Wrenn, 1971).

#### Hormonal Regulation of Oviductal Epithelial Cytodifferentiation

Animal species differ in the degree of oviductal differentiation that occurs prior to puberty. The oviductal epithelium of humans (Woodruff and Pauerstein, 1969) and guinea-pigs (Price, Zaaier and Ortiz, 1969) already contains ciliated and secretory cells at the time of birth. Cytodifferentiation of the oviduct of rats (Kellogg, 1945) and rabbits (McCarron and Anderson, 1973) occurs after birth but prior to the onset of maturity. The oviductal epithelium of the beagle bitch however remains undifferentiated until puberty. With the initiation of cyclicity the oviductal epithelium of the bitch undergoes a complete differentiation during the proestrus stage followed by a subsequent de-differentiation during metestrus and anestrus. This pattern is repeated during each subsequent estrus cycle (Verhage et al., 1973a).

The main body of evidence implicating estrogen in the initial cytodifferentiation of the oviductal epithelium has come from studies involving the administration of estrogen to prepubertal animals. As already mentioned, the administration of estrogen alone to young (4-7 days) chicks for approximately ten days will result in the differentiation of the primitive mucosal cells into tubular gland, goblet and ciliated cells (Kohler, Grimley and O'Malley, 1969; Palmiter and Wrenn, 1971). A similar study has been performed by Verhage and his colleagues on the prepubertal beagle pup (Verhage et al., 1973b). These authors also found that administration of estradiol to the immature animal resulted in a complete cytodifferentiation of the oviductal epithelium. Using ultrastructural criteria they concluded that the induced differentiation was identical to that which occurred normally in the cycling bitch.

Injections of estradiol given to immature rhesus monkeys will also provoke the appearance of an 'adult' oviductal epithelium (Brenner, 1973). In addition the oviductal epithelium of the fetal rhesus monkey has been observed to undergo differentiation at approximately 150 days gestation, with a subsequent de-differentiation during the neonatal period. Fetal ovariectomy at 120 days gestation prevents the wave of differentiation seen at 150 days, and it is assumed that the fetal ovaries are secreting enough estrogen during the period of 120-150 days gestation to provoke the prenatal cytodifferentiation (Ibid.).

Although conclusive studies have not implicated estrogen in the initial differentiation of the oviductal epithelium of the human and guinea-pig, there is evidence that the reproductive tracts of these species are exposed to estrogenic stimulation during fetal life

(see Dallenbach-Hellweg, 1971 p41; and Price, Zaaier and Ortiz, 1969). Whether this stimulation occurs at the time the oviductal cells are undergoing differentiation, and whether the estrogen is instrumental in the differentiation process in these species is still not clear.

The presence of estrogen receptor molecules in the oviductal tissues and their ability to transfer estradiol to the nucleus has been demonstrated in fetal rats of twenty-two days gestation by dry-mount autoradiography (Nakai et al., 1972). However estrogen synthesis cannot be detected in the rat ovary prior to 8-14 postnatal days (Cierciorowska and Russfield, 1968; Presl et al., 1965). The ability of a target tissue to respond to estradiol is dependent on the presence of the protein receptor molecules. Clark and Gorski (1970) deduced from the results of their experiments that the synthesis of receptor molecules was an autonomous property of uterine cells. However they based their conclusion on the fact that early ovariectomy did not affect the development of binding sites in the rat uterus. This does not, however, eliminate the possibility that extra-ovarian sources of estrogen are stimulating receptor synthesis and indeed Weisz and Gunsalus (1973) have detected relatively large amounts of plasma estrogen in newborn and immature female rats which they believe is of adrenal origin. There is now considerable evidence which implicates estradiol in the regulation and even synthesis of its cytoplasmic receptor molecules (see Brenner and West, 1975 for review; Cidlowski and Muldoon, 1974).

In both the rat and the chicken, estradiol receptor molecules have been detected in the oviduct prior to the cytodifferentiation of the epithelium (Nakai et al., 1972; Teng and Teng, 1975a&b, 1976).

It has already been demonstrated that estrogen administration can stimulate the differentiation of the chick oviductal cells. Until further experiments are performed it can only be assumed that the presence of receptor molecules in the undifferentiated rat oviductal epithelial cells indicates that these cells are capable of selectively retaining estradiol. The question of whether estradiol initiates the cytodifferentiation of these cells in the rat and other species must as yet remain unanswered.

#### Pathological Effects of Estrogen on the Female Reproductive Tract

Soon after its isolation, it was discovered that, in addition to its physiological growth-promoting effects, estrogen could have a pathological influence on its target tissues, especially when administered unremittingly for long periods of time. Abnormal responses to exogenous estrogen were often observed in the uterus (see Parkes and Deanesly, 1966 and Zondek, 1941 for reviews). These included cystic glandular hyperplasia, squamous metaplasia of the uterine epithelium, and varying degrees of infection ranging from a mild endometritis to a severe pyometra (Bo, 1957; Crossen and Loeb, 1944; Iglesias and Lipschutz, 1946; Korenchevsky and Hall, 1940; Morell and Hart, 1941a&b; Nelson, 1937; Zondek, 1936).

The most commonly described response of the oviduct to prolonged estrogenic stimulation was the development of infection leading to pyosalpinx. Hale (1944) reported a high incidence of bursal and oviductal inflammations in both young and mature rats treated with estrogen. Following injections of testosterone to infantile female rats, a procedure which leads to the development of polycystic ovaries, Bradbury (1941) also found tubo-ovarian abscesses to be a

common complication. These 'masculinized' rats showed diestrus vaginal smears for two weeks following the cessation of the testosterone treatment. Thereafter the animals exhibited continual vaginal estrus, suggesting the unopposed secretion of estrogen. Oviductal abscesses were also reported by Everett (1939) in a strain of albino rat that spontaneously exhibited a state of persistent estrus.

The only other mention in the literature of the response of the oviduct to continual estrogen stimulation is by Meissner, Sommers and Sherman (1957). These authors noted dilation of the oviduct, with moderate epithelial hyperplasia in two-thirds of their rabbits treated with the synthetic estrogen stilbesterol. The rest of the treated animals exhibited inflammatory changes.

In contrast, the endometrium of these rabbits appeared to be more reactive to the estrogen. The uteri of six out of eighteen stilbesterol treated rabbits developed endometrial carcinoma, and endometrial hyperplasia and other abnormal conditions were found in many more of the treated animals. None of the control rabbits exhibited carcinoma or other uterine or oviductal abnormalities.

### Estrogen and Cancer

Meissner and his colleagues were not the first to notice an association between estrogen treatment and the subsequent development of cancer in target tissues. In 1932, Lacassagne (see Lacassagne, 1955) published an account of the development of mammary cancer in male mice receiving injections of estrone. Since then, this malignant response following experimental estrogen treatment has been observed in a variety of tissues and species (see Botella-

Llusia, 1973 for review).

In addition to the experimental evidence, there is strong clinical evidence for an association between uterine carcinoma and exposure to excess amounts of estrogen. Certain theca and granulosa cell tumours of the ovary are found to secrete relatively large amounts of estrogen and proliferative and hyperplastic conditions of the endometrium are commonly observed in patients with these tumours (Targett, 1974). In a review of the literature concerning estrogen-secreting tumours of the ovary, Greene (1957) found that 10-27% of these tumours were associated with endometrial cancer, and Botella-Llusia (1973) observed that endometrial carcinomas were 17-20 times as common in women with 'feminizing' tumours as in the general population.

An analogous condition can be observed in the laboratory rabbit which as mentioned before, remains in persistent estrus unless mated. Burrows (Burrows and Horning, 1952) found a remarkably high incidence of endometrial carcinoma in his unmated stock rabbits and attributed this to the state of prolonged estrus. King (1973) reports that adenomatous hyperplasia is uncommon in most animals with the exception of the rabbit. He also reports that adenocarcinoma of the uterus occurs commonly in this species after two years of age and constitutes the single most common neoplasm in the rabbit.

Recent medical reports have indicated an ominous correlation between hyperplastic and malignant conditions of the endometrium and the therapeutic use of estrogens in human patients (Østergaard, 1974; Ziel and Finkle, 1975). Smith and his colleagues (Smith et al., 1975) have noted that there is a rising trend towards the development of endometrial carcinoma in estrogen users that do not otherwise

possess the constitutional characteristics previously associated with the disease (e.g. obesity and hypertension).

However the actual role of estrogenic molecules as carcinogens is still disputed. Although estrogen was first implicated as a carcinogenic agent in the development of mammary carcinoma, some researchers now believe that, at least in rodents, the role of estrogen in the development of this tumour is an indirect one mediated through its regulation of prolactin secretion (Beuving and Bern, 1972; Meites, 1972). In his review of the role of estrogen in genital tumourigenesis, Botella-Llusia (1973) relates several lines of work which suggest that some intermediate metabolite of the estrogen molecule which resembles known carcinogens is responsible for the neoplastic transformation. He concludes however, that the bulk of present evidence suggests that estrogen acts primarily as a catalyst or accelerator of the proliferation of cells made neoplastic by some other oncogenic agent.

Nevertheless evidence has accumulated in the last ten years concerning one particular kind of genital neoplasm that implicates estrogen directly as a causative agent. In the late 1960's several clinicians noticed a sudden sharp rise in the incidence of adenocarcinoma of the vagina in young women (approximately 15-22 years of age). Analysis of these cases led to the discovery that the mothers of a highly significant proportion (approximately 95%) of those girls who developed adenocarcinoma had received estrogen therapy during the early months of pregnancy. Stilbesterol, or other nonsteroidal synthetic estrogens, had been prescribed for these women, usually to prevent a threatened abortion (Greenwald et al., 1971; Herbst, Ulfelder and Poskanzer, 1971; Herbst et al., 1974; Ulfelder, 1973).



Forsberg (1972, 1973) has examined the possible role of estrogen in the development of vaginal adenocarcinoma, using the mouse as a model. In this species, both the urogenital sinus and the Müllerian ducts take part in vaginal development. Forsberg has shown that the primitive Müllerian epithelium is capable of giving rise to the stratified squamous epithelium of part of the adult vagina. This transformation, i.e. from pseudostratified Müllerian epithelium to the stratified vaginal epithelium occurs shortly after birth in the mouse.

Forsberg found that injections of either estradiol-17 $\beta$  or stilbesterol given to neonatal mice during the first five days after birth inhibited the transformation process in the region of the vaginal fornices and the uterine cervix. This untransformed epithelium then persisted into adult life and eventually, estrogen secreted by the mouse's own ovaries caused the proliferation of this epithelium into glandular downgrowths. Forsberg found that although ovariectomy of the mice one month after the neonatal hormone treatment had no effect on the distribution of the untransformed epithelium, it did prevent the subsequent development of the glandular downgrowths.

Forsberg has proposed that the stilbesterol received by the mothers during the early months of pregnancy may be inhibiting a similar 'transformation' process that occurs in the primitive Müllerian duct of the human fetus. As already mentioned, the adult human vaginal epithelium does not contain any cells that are derived from Müllerian epithelium. However the Müllerian duct is involved in earlier stages in the embryological development of the vagina.

In addition, comparison of the vaginal adenocarcinomas with other genital tract neoplasms and evaluations of their morphology have convinced many pathologists that the growths have a Müllerian rather than a sinus or Wolffian origin (Silverberg and DeGiorgi, 1972).

An estrogenic stimulus has thus been associated with the development of carcinoma in two types of epithelium derived from the Müllerian duct, i.e. the epithelium of the endometrium and, if Forsberg's hypothesis is correct, primitive Müllerian epithelial remnants found in the vagina. It is interesting, therefore, that in all of the studies involving long-term estrogen treatment, none of the experimental animals have developed carcinomas of the oviductal epithelium. Nor has oviductal carcinoma been observed clinically in conjunction with estrogen-secreting tumours of the ovary, although many of the oviducts in these cases exhibited hyperplastic changes (Dougherty and Cotten, 1964; Pauerstein and Woodruff, 1966).

Two studies have been made of the oviductal mucosa in cases of human endometrial carcinoma. Rewell and Towers (1956) found atrophic, flattened epithelium in the oviducts of thirty-three out of fifty cases of endometrial carcinoma, with the remainder of the oviducts exhibiting epithelium typical of various stages of the normal cycle. The oviducts from the postmenopausal patients of the control group however exhibited atrophic epithelium in less than half of the cases (i.e. eighteen out of forty-three). They proposed that persistent and prolonged hyperestrinism would prevent the postmenopausal atrophy of the oviductal epithelium and therefore from their data concluded that hyperestrinism was not an important etiological factor in the development of the uterine neoplasms.

A more recent report however, comes to a completely different conclusion. Dallenbach-Hellweg and Rom (1970) found that the oviducts of fifty postmenopausal patients with endometrial carcinoma exhibited excessive epithelial hyperplasia. The oviducts of their control patients of equivalent age but without endometrial neoplasms showed atrophic epithelium. From their results they concluded that a common hormonal stimulus was responsible for both the hyperplastic and neoplastic changes.

Primary oviductal cancer is very rare, with the reported incidence varying from 0.16 to 1.81% of all primary malignancies of the female genital canal (Woodruff and Pauerstein, 1969). In light of current evidence supporting a link between excess estrogenic stimulation and the development of uterine carcinoma, one could hypothesize that the rarity of oviductal malignancy was in part due to its relative resistance to the 'tumour' promoting effects of estrogen. With this in mind it was decided to re-examine the effects of long-term estrogenic stimulation on the oviductal epithelium

#### Aims of Present Study

The emphasis of this study has been placed on the morphological changes elicited by estradiol in the oviductal epithelium. At the present time, one of the main criteria for pathological change is alterations in morphology. It has also been shown that some of the molecular events initiated by estrogen within the target cells are visible at the ultrastructural level. For this reason, most of the experimental material has been examined with the electron microscope.

This study was initiated with a re-examination of the oviductal

epithelium of the mature rat and rabbit. This was done to establish a picture of the epithelium under the physiological influence of the animals' own ovarian estrogen, which could then be used as a baseline for comparison.

The findings of Verhage and his colleagues on the beagle and the examination by several research units of the estrogen-induced cytodifferentiation of the chick oviduct has shown that the pattern of estrogen action in the mature animal can be followed by administering the hormone to the immature animal. Therefore the second part of this study was devoted to a repetition of the study performed on beagle pups (Verhage et al., 1973b) using immature rats and rabbits.

The third part of this study centered on the long-term effects of estradiol on the oviductal epithelium of the rat and rabbit. A desire to compare these results to the clinical situation, and to earlier studies, necessitated the use of intact animals. Obviously the administration of estradiol to the intact animal has profound effects on the hypothalamico-pituitary-ovarian axis. The effects of the consequent alterations in endogenous hormonal output were included in the analysis of the changes observed in the oviductal epithelium.

Several factors encountered during the work that is described in Parts II and III of this report pointed to the difficulty in interpreting the effects of hormonal administration to intact animals.

Ovariectomy alone is insufficient to eliminate the interference of endogenous hormones, and a more radical excision of endocrine organs necessarily introduces more variables into the system. Therefore, following the completion of the work described above, the possibility

of using an organ culture system for the study of the effect of short- and long-term estrogen treatment on the oviductal epithelium was examined.

Although a few pilot hormonal studies were performed, there was only a small amount of time left for experimental work. Therefore the bulk of the organ culture work was an assessment of the functional viability of the rat oviductal epithelium using morphological criteria established in the first part of this study. The results of these experiments, including an examination of the morphology of the rat oviduct in organ culture and a review of previous work utilizing the oviductal organ culture system, can be found in the Appendix of this report.

## MATERIALS AND METHODS

### I. GENERAL METHODS AND TISSUE PREPARATION TECHNIQUES

#### Animal Care and Treatment

All of the animals were kept in the licensed animal house in the Bute Medical Building of St. Andrews University. The temperature of the animal house was maintained at approximately 24°C and the animals were subjected to twelve hours of light and twelve hours of dark per day.

All of the experimental rabbits were obtained commercially, from Hylyne Commercial Rabbits (Hartford, Northwich) and were quarantined for one week before being approved as fit for experimental procedures. A few animals used solely for anatomical studies and preliminary ultrastructural examinations were obtained from the stock of rabbits kept in the animal house. With the exception of the six-week-old animals which were initially kept in pairs, the rabbits were housed in separate cages and all received commercial animal food (Diet 41B, Farmers Co-op Aberdeen) and water ad libitum.

The rats used for the second long-term experiment were obtained from A. Tuck and Son (Rayleigh, Essex). All of the rest of the rats used were stock Sprague-Dawley albinos bred in the animal house. The three-week-old rats were housed with their mothers until they reached five weeks of age. All of the rest of the rats were caged in groups of 2-4 of the same age. The rats were subjected to the same heat, lighting and food conditions as the rabbits, although housed in a separate room.

Any operative procedures performed on the animals were carried out in aseptic conditions in the operating room of the animal house.

It is now appreciated that the experimental error inherent in weighing out such small amounts of estradiol would have been reduced if much larger quantities had been used to make up a large volume of stock solution.

In the immature rat experiment the method of weighing of estradiol was, in fact, improved in that 2000  $\mu$ g of estradiol benzoate was dissolved in 200 ml corn oil; but in the immature rabbit experiments the possibility of there being variation in the dose received by individual animals would have been reduced if the improved weighing technique had been used ( see p 113 and p 2 of addendum).

Ovariectomy was performed via bilateral flank incisions under Nembutal (Abbott) anaesthesia whereas local anaesthesia (subcutaneous injection of 0.15ml Xylocaine (Duncan, Flockhart and Co. Ltd., Edinburgh)) was used for subcutaneous implantation of estradiol pellets. All operative procedures were carried out under Home Office license (ED 1897).

#### Estrogen Preparations

The estrogen used in single subcutaneous injections was prepared by dissolving 200 $\mu$ g of  $\beta$ -estradiol-3-benzoate (Sigma) in 20mls of corn oil, thus giving a final concentration of 1 $\mu$ g estradiol per 0.1ml vehicle. In the long-term estrogen experiments, 15-20mg sterile pellets of estradiol BPC (Organon) were used for implantation. The preparation of estrogen used in organ culture experiments is described below.

#### Tissue Processing

In a study such as the present one where the results are based mainly on ultrastructural evidence, it is imperative that the fixation of the tissues is of the highest quality in order to reduce the chances of making judgements based on artifact. Each different tissue studied presents different problems as regards fixation, and therefore the best fixative must be found for each tissue under investigation.

As is usual in these situations the search starts with whichever fixative is used routinely in the researcher's department. The initial solutions therefore tested were 2% Glutaraldehyde in 0.08M Sodium Cacodylate buffer pH7.3, with postfixation in 1% OsO<sub>4</sub> in Cacodylate buffer. Prior to the start of the present investigation it had been observed that the commercially obtained Glutaraldehyde (Taab-designated specifically for electron microscopy) was giving unsatisfactory



results. A sample of the Taab Glutaraldehyde was subjected to analysis in an ultraviolet spectrophotometer and two absorption peaks were found, one at 280nm and one at 235nm. According to Hayat (1973), an  $\alpha$ - $\beta$ -unsaturated dimer of Glutaraldehyde forms under ordinary storage conditions, and along with other impurities this polymer, which absorbs in the ultraviolet at 235nm, can inactivate enzymes in the tissues. 'Pure' Glutaraldehyde should show only one absorption maximum at 280nm and Hayat therefore recommended purifying the commercial solution. As a result, all Glutaraldehyde used in our laboratory was purified with activated charcoal and distilled according to the method of Fahimi and Drochmans (1965).

Glutaraldehyde treated in this way proved unsatisfactory for the oviduct, however, as most of the mitochondria of the epithelial cells exhibited 'ballooning' or were 'exploded'. In their investigations, Webster and Ames (1969) also observed mitochondrial swelling with a 3% Glutaraldehyde fixing solution. It was then realized that purification of the Glutaraldehyde had decreased the solution's osmolarity, a property which can greatly influence the resulting morphology. Hayat (1970) notes that the molarity (or tonicity) of a 3% solution of commercially supplied Glutaraldehyde may be as high as 570 milliosmoles, whereas 3% purified Glutaraldehyde has a tonicity of only 300 milliosmoles. A slightly hypertonic solution (400-450 milliosmoles) of buffered Glutaraldehyde is recommended by Hayat for general fixation.

A series of experiments were then undertaken in an attempt to raise the osmolarity by varying the concentration of Glutaraldehyde and the buffer type and by adding the non-electrolyte sucrose. The following fixing solutions were tested on pieces of

rabbit oviduct with no improvement in morphology:

2% Glutaraldehyde in 0.1M Phosphate buffer with 0.22M Sucrose, pH 7.4

2% Glutaraldehyde in 0.1M Phosphate buffer, pH 7.4

2% Glutaraldehyde in 0.08M Sodium Cacodylate buffer with 0.22M Sucrose, pH 7.3

2% Glutaraldehyde in 0.08M Sodium Cacodylate buffer, pH 7.3

6% Glutaraldehyde in 0.08M Sodium Cacodylate buffer, pH 7.3

It was then decided to try Karnovsky's (1965) high osmolarity (approx. 2010 milliosmoles) Formaldehyde-Glutaraldehyde fixative. The high quality of preservation obtained with this solution resulted in its being adopted as the routine fixative for these experiments.

After fixation for 2-2½ hours in 4% Paraformaldehyde 5% Glutaraldehyde in 0.08M Sodium Cacodylate buffer, pH 7.3 (in Karnovsky's method, Formaldehyde is prepared from Paraformaldehyde powder to minimize the impurities) the tissue blocks were rinsed for fifteen minutes in three washes of 0.08M Sodium Cacodylate buffer, pH 7.3 before postfixation in 1% OsO<sub>4</sub> in the same buffer. Following osmication for one hour and a further buffer rinse, the blocks were dehydrated in graded alcohols, cleared in epoxy propane and embedded in either Araldite or Spurr resin. All processing was carried out at room temperature.

One-micron-thick sections were cut from the plastic-embedded material on a Reichart OMU3 ultramicrotome and stained on glass slides with 0.25% Toluidine Blue containing 0.5% Sodium Tetraborate. Pale gold to silver ultrathin sections cut on the same microtome and

placed on copper grids were routinely stained with Uranyl Acetate and Lead Citrate and examined in a Zeiss EM 9S electron microscope. Silver staining for ribosomal proteins was performed on ultrathin sections mounted on titanium grids according to the method of Smith and Stuart (1971).

Lysosomal acid phosphatase was demonstrated in tissue processed for electron microscopy by a revised Gomori method. Tissue fixed in the Paraformaldehyde-Glutaraldehyde solution was rinsed overnight in 0.08M Sodium Cacodylate with 7.5gms% Sucrose, pH7.3, at 0-4 °C. The blocks were then placed in substrate (0.12g Lead Nitrate dissolved in 100mls Sodium Acetate/Acetic Acid buffer pH 5.3 + 10mls distilled water containing 0.3g Glycerophosphate (Sigma)) which had been brought to 37°C for one hour prior to incubation. After 3-4 minutes incubation the blocks were rinsed in Cacodylate buffer, postosmicated, and then processed to Araldite in the usual manner. Unstained ultra-thin sections were then examined in the electron microscope.

Material for light microscope examination was fixed in 10% Formol Saline and processed to paraffin. Seven-micron sections were routinely stained with Haematoxylin and Eosin or 0.1% Alcian Blue in 3% Acetic Acid. Sections of pituitary were stained with Iron PAS-Orange G (Pearse, 1953).

#### Cell Measurement Techniques

Three non-serial sections were cut from Araldite blocks representing each rat in the first part of the experiment described in Part II of the results. These were stained with Toluidine Blue and permanently mounted for cytomorphometric measurements. The height and width of approximately twenty oviductal epithelial cells

and their nuclei were measured for each animal, and if possible ten ciliated and ten nonciliated cells were chosen. Cell measurements were made only on sections of ampulla, as the preservation of the epithelium in this region of the oviduct was better than that found in the isthmus, and the arrangement of the mucosal folds in this region resulted in less distortion of the cells. Only cells which were clearly sectioned through the longitudinal plane were measured. Cell and nuclear volumes were calculated using the formula  $\pi r^2 h$ , as used by Verhage et al. (1973a&b). The diameters of thirty nucleoli were also measured. The area of each slide used for nucleolar measurement was chosen randomly, but all of the nucleoli in that area were measured until the quota of thirty was reached to prevent any unconscious size bias in choosing nucleoli. All measurements were made on a Hilux 70 microscope using a screw micrometer eyepiece calibrated for a X90 oil objective.

In the second part of the experiment described in Part II of the Results, cell and nuclear measurements were made on light micrographs of sections of the oviducts of the immature rabbits. These measurements were made using fine dividers and a millimeter ruler. Cell and nuclear measurements were made on micrographs with a final magnification of X600. Nucleolar measurements were made on micrographs having a final magnification of X2400.

It was considered justifiable to use different techniques on the grounds that the aim of the experiment was to discern variations between animals of the same species caused by the different estrogen treatments. Only the patterns of change were compared among the rat,

rabbit and pup (Verhage et al., 1973b) results, and not the definitive values obtained for the nuclear and cell volumes.

### Organ Culture

The culture medium used in all experiments was Minimum Essential Medium with Earles salts combined with Sodium Bicarbonate (7.5% w/v) (Flow Laboratories). Penicillin and Streptomycin (BDH) were added to the medium giving a final concentration of 88U each per ml. Glutamine and calf serum (Flow) were stored at  $-20^{\circ}\text{C}$  and following incubation at  $37^{\circ}\text{C}$  were combined with the medium to give final concentrations of one and ten percent respectively.

The rat oviduct was teased apart and short lengths of the oviduct were cut and placed on squares of cellulose acetate paper. Each oviduct provided 5-9 explants. Trowell's grid method (Moscana, Trowell and Willmer, 1965) was employed to support the pieces of oviduct at the surface of the medium. The petri dishes containing the medium and grids were placed in McIntosh and Fildes jars (Gallem Kamp) and gassed with 95%  $\text{O}_2$  5%  $\text{CO}_2$  at three psi. The jars were then incubated at  $37^{\circ}\text{C}$ . They were regassed and the medium changed every two days.

A stock solution of estradiol was made by dissolving 10mg estradiol BPC (fused implants-Organon) in 10ml of sterile millipore-filtered 75% ethanol. One-tenth of a ml of the stock solution was added to 100mls of medium, giving a final concentration of  $1\mu\text{g}$  estradiol per ml medium. Sterile millipore-filtered 75% ethanol was added to medium in control cultures in a final concentration of 0.1%.

Cultured oviduct segments were processed for electron microscopy following the same procedure as described above. Specimens

for light microscopy were fixed in Bouin's Picric Acid fixative, as the yellow colour imparted to the tissues facilitated the handling of the tiny pieces. Paraffin sections were stained routinely with Haematoxylin and Eosin.

## II. EXPERIMENTAL PROCEDURES

### A. Morphology of the Rat and Rabbit Oviductal Epithelium (Results and Discussion-Part I)

The morphology of the oviduct was studied at the light and electron microscopic level in the rat at three weeks, four months, eight months and fifteen months of age, and in the rabbit at two months, four and a half months, ten months, fourteen months and eighteen months. The mature rabbits were all sacrificed during estrus. Vaginal smears were taken from the four-month-old rats daily for several cycles before they were sacrificed at the proestrus stage of the cycle. All of the animals served as controls in the experiments described below in B and C (First Long-Term Experiment).

### B. The Effects of Short-Term Estradiol on the Epithelium of the Immature Rat and Rabbit Oviducts (Results and Discussion-Part II)

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in 0.1ml corn oil for 1-6 days. All animals were sacrificed twenty-four hours after their last injection.

Oviductal tissue from all animals was processed for electron microscopy. One-micron thick Araldite sections were prepared for cytomorphometric studies as described above. The uteri of all of the rats were weighed at the time of sacrifice. The ovaries were routinely processed to paraffin, but unfortunately owing to the extremely small size of the immature rats' ovaries they were lost during the processing procedure.

ii. Rabbit Experiment:

In this experiment three litters each of four eight-week-old rabbits were used. Four of these animals were used as controls; two intact controls received 0.1ml corn oil for one and three days and the other two rabbits underwent bilateral ovariectomy and were sacrificed one and two weeks postoperatively. The rest of the immature rabbits received daily subcutaneous injections of  $1\mu\text{g}$   $\beta$ -estradiol-3-benzoate in 0.1ml corn oil for 1-6 days. (Note: one, two, five and six days- one rabbit; three and four days- two rabbits.) Due to the long interval between the arrival in the animal house of the three different litters, the estrogen was prepared in three batches, one for the experimental animals in each litter. All rabbits were sacrificed twenty-four hours after their last injection.

Oviductal tissue from all rabbits was processed for electron microscopy. Light micrographs were taken from one-micron Toluidine Blue stained sections of the rabbit oviducts. The uteri and ovaries of the rabbits were weighed at the time of sacrifice and the ovaries were processed to paraffin for light microscopy.

iii. Presentation of Results:

The effects that the estrogen had on the cell volume, nuclear volume, nucleo-cytoplasmic ratio and nucleolar diameter of the immature rat oviductal epithelium can be seen in Table I and Figures 26, 27 and 28 (Results and Discussion-Part II). There were no significant differences between the ciliated and nonciliated cells in any of the various parameters measured in any animal and therefore the results for the two cell types were combined. There were also no significant differences between the two rats treated with oil only for one and six days and consequently these results were combined to give the control values. The effect that the estradiol treatment had on the uterine weight of the rats is illustrated in Figure 29.

The effects that the administration of estrogen to intact eight-week-old rabbits had on cell volume, nuclear volume and nucleolar diameter can be found in Table II and Figures 36, 37 and 38 (Results and Discussion-Part II). Again, values for ciliated and nonciliated cells were combined. Although there was a significant difference ( $p < 0.05$  by t-test) between the average nuclear volumes obtained for the two control rabbits, the results of these animals were combined in order that a value expressing the variation seen in the controls would be used when comparing to the experimental results.

Differences were also observed between the two animals treated for three days with estradiol and the two animals treated for four days. These results were not combined however, as the two rabbits treated for three days and the two rabbits treated for four days



were in different litters and therefore may not have received identical dosages of estradiol. As already mentioned, fresh estradiol/oil mixtures were prepared for each rabbit litter. Because of the very small amount of estrogen being weighed out on each occasion (i.e. 200 $\mu$ g) it is quite possible that the final dosages that the animals in the different litters received could have varied considerably from the proposed daily dosage of 1 $\mu$ g estradiol.

The uterine weights of all control and experimental rabbits can be found in Figure 39. These data also suggest that there were differences in the daily dosage of estrogen received by the experimental animals in the three litters.

C. The Effects of Long-Term Estrogen Administration on the Epithelium of the Rat and Rabbit Oviducts (Results and Discussion-Part III)

i. First Long-Term Experiment:

Twelve rats were implanted subcutaneously with 15mg pellets of estradiol. According to the manufacturer (Crystal Implantation, Organon Laboratories Ltd.) estradiol is absorbed from these pellets at an approximate rate of 75 $\mu$ g per day. Four of the rats were implanted at three weeks of age, four at three months and four at approximately eighteen months.

Seven rabbits were implanted with the estradiol pellets. One was implanted at seven weeks of age, one at ten weeks, and the rest at approximately sixteen weeks of age. Two of the rabbits died over the weekend and as no tissues were salvaged, these animals were not included in the results.

The animals in this experiment were sacrificed when they were found to be in extremis. Pieces of mammary gland, vagina, uterus

and the ovaries and pituitary of each animal were processed for light microscopy. Samples of oviduct were processed for both light and electron microscopy. The oviducts of those animals which were found dead and also those exhibiting pyosalpinx were processed to paraffin only.

ii. Second Long-Term Experiment:

The epithelium of the rat oviducts in the first long-term experiment often showed 'proliferative' changes which were assumed to be caused by the estrogen treatment. As these changes were observed as early as three months following estrogen implantation, and as no rats were examined at an earlier stage, the second long-term experiment was initiated to analyze the sequence of events in the oviduct in the first few months following implantation of the pellets.

Sixty-four 200-250gm virgin female Sprague-Dawley rats were used in this experiment. Forty of the rats were implanted with estradiol pellets of approximately 15mg. The remaining twenty-four rats served as controls.

Sixteen rats (ten experimental and six control) were sacrificed following one, two, three and four months of estrogen treatment. The body weight of each animal was obtained prior to sacrifice. Following sacrifice the ovaries, oviducts, uterus and pituitary were removed, dissected free of all connective tissue, blotted dry and weighed. (Note: oviducts exhibiting large pyosalpinx and uteri exhibiting pyometra were not weighed or included in the calculations.) Incisions were made in each of the uterine horns to allow any fluid to escape before weighing. This also facilitated fixation of the uterus.

Following weighing, all of the tissues were fixed in Formol Saline and processed to paraffin. Sections of the ovaries, oviducts and uteri were stained with Haematoxylin and Eosin. Sections of oviducts and uteri were also stained with Alcian Blue. Sections of pituitary were stained with Iron PAS-Orange G. By this technique, alpha-cell granules stain an orange colour, beta-cell granules are PAS +ve red and delta-cell granules are PAS +ve purple. (Note: In part III of Results and Discussion the results for both the rat experiments are combined.)

#### D. The Rat Oviductal Epithelium in Organ Culture (Appendix)

The introduction of the technique of organ culture into this research project was not entirely unrelated to the recent establishment of a tissue culture suite in the department. Unfortunately, the rapidly diminishing amount of time available for this research prevented the comprehensive series of pilot studies necessary to establish the optimum conditions for culture of the rat oviduct. The survival of several explants with good preservation of the epithelium for 6-9 days using the technique described in Part I of the Materials and Methods (a technique used in the majority of experiments being performed in the tissue culture suite at the time) resulted in this method being used in all of the experiments performed using the rat oviduct. But it was by no means certain that this method was the best of those presently available.

Six organ culture experiments were performed. The first two experiments were pilot studies and used the oviducts of three rats. Explants were fixed following two, four, five, six and eight days of culture and processed for light microscopy.

Explants from the third organ culture experiment were processed for electron microscopy. The oviducts of four rats were used and explants were examined after two, four, six and nine days in culture. Half of the explants were cultured in medium without serum.

Explants from one oviduct of a rat that had received estradiol (from a subcutaneous pellet) for eighty days were cultured in the fourth experiment. Pyosalpinx involved the other oviduct of the rat. Light microscopic examination of the explants in this experiment revealed the presence of fibrous tissue replacing the epithelium of the lumen, and also the presence of inflammatory infiltrate in the other layers, suggesting that this oviduct had also been the site of infection. The lack of an intact epithelium in this experiment meant that it was not possible to examine the behaviour of the epithelium following isolation from the source of long-term estrogen stimulation.

In experiments five and six the effect of adding estradiol to the medium was tested. Six rats were used in experiment five and four of the rats had had estrogen treatment for four months. Explants from one oviduct of each rat were cultured in medium containing estradiol (1 $\mu$ g/ml medium), and explants from the other oviduct were cultured in the absence of the hormone. Explants were processed for light microscopy following two, four, six and nine days in culture.

The survival of the explants in experiment five was not as good as that which occurred in the earlier experiments. This was possibly due to the fact that serum had not been added to the medium in this experiment because of the possibility that it contained free

hormones. Therefore the medium of all cultures in experiment six contained serum.

Four rats provided oviductal tissue in the sixth organ culture experiment. Half of the explants were cultured in the presence of estradiol ( $1\mu\text{g/ml}$  medium). Explants were processed for electron microscopy following two, four, six and nine days in culture.

## RESULTS AND DISCUSSION

### I. MORPHOLOGY OF THE RAT AND RABBIT OVIDUCTAL EPITHELIUM

#### Anatomy of the Rat and Rabbit Oviduct

The anatomical relationship of the oviduct to the rest of the reproductive tract in the rat and the rabbit is shown in Figure 1. Both animals have a bicornuate uterus, with each uterine horn ending in a cervix in a common vagina. Anatomically, however, the oviducts of the two species are very different.

The rat oviduct is very tightly coiled, and at first, is difficult to distinguish visually from the ovary. This is partly due to the fact that the oviductal ostium opens into a bursa, the bursa ovarica, and the epithelium of the fimbriated margin of the oviduct is continuous with the mesothelium lining the bursa. The ovary invaginates into the bursa and there is a transition from bursal mesothelium to the ovarian germinal epithelium near the hilum of the ovary. Normally there is only a very small amount of fluid within the bursa, and the oviductal coils are closely applied to the surface of the ovary. In contrast, the rabbit oviduct is relatively straight and there is no bursa interposed between the oviduct and ovary. Instead the fimbriae expand in a fan-shaped manner along the dorsal aspect of the ovary and, as is the case in the human oviduct, the opening is continuous with the peritoneal cavity.

#### Histology of the Oviduct

It is convenient for descriptive purposes to divide the oviduct into the four segments proposed by Nilsson and Reinius (1969). Sections of the tightly coiled rat oviduct provide very characteristic

profiles of these regions (Fig. 2a). Starting at the ovarian end of the oviduct, the preampulla includes the infundibulum and its fimbriated margin, which curves back on to the outer surface of the oviduct for a short distance. The epithelium lining this part of the oviduct is composed almost entirely of ciliated cells.

The ampulla has a higher percentage of nonciliated cells, which increase in number as the ampullary-isthmic junction is approached. The epithelium of the preampulla and the ampulla is thrown into highly developed folds, each with a core of lamina propria. The epithelial cells on these folds are characteristically one cell layer thick on the tips of the folds, but as one moves down the sides of the fold more complex patterns are found, with the epithelial cells forming gland-like spaces (Fig. 2c). At times this gives the epithelium a stratified appearance, but closer inspection reveals that all of the cells have one surface facing the lumen. The ampullar segment in the region of the eggs and cumulus cells is very distended and the folds are more widely spaced (Fig. 2b).

The isthmus comprises the bulk of the rat oviduct and again has a very characteristic profile with a much narrower diameter. The immersion fixation procedure gives an artifactually distended lumen, which more or less disappears when a perfusion technique is used (Fig. 2d, c.f. Fig. 2a). The epithelial cells project into the lumen in pseudo-papillae, with crypts between projections. These crypts are shown to contain ciliated cells, whereas the majority of the isthmic cells are nonciliated (Figs. 2d & 4).

The final segment of the rat oviduct is the junctura (Fig. 2e). The epithelial cells here appear to be slightly taller than in the

other segments, and the folds have a very characteristic squared appearance.

Sections of the rabbit oviduct on the other hand, do not present such varying profiles. The ampullar and isthmus regions each occupy approximately half of its length and differ chiefly in number and height of folds, diameter of lumen and thickness of muscle wall (Fig. 3a&b). The most important point to note concerning the low power architecture of the mature rabbit oviduct is that the epithelium dips down into pits between folds, with the result that in certain sections these pits give the appearance of subepithelial glands (Fig. 3c).

#### Ultrastructure of Oviductal Epithelial Cells

i. Nonciliated Cells. The columnar epithelium of the oviduct is composed of ciliated and nonciliated cells. The most common type of nonciliated cell in the rat is found in the isthmus, which as mentioned before occupies most of the length of the oviduct in this species. This type of cell (Figs. 4, 5a&b) is characterised by long microvilli ( $\sim 4-6 \mu$ ) (often called stereocilia) and by dark granules in the apical cytoplasm. These granules often contain areas of varying density suggesting a heterogeneous composition (Fig. 5b). Rough endoplasmic reticulum is present to varying degrees, and in some cells, especially in the proestrus oviduct, is widely dilated (Fig. 5a). Nonciliated cells in other regions of the rat oviduct differ mainly in that they possess shorter microvilli ( $\sim 2 \mu$ ) and fewer or no granules (Fig. 7-NC).

Another feature of the supranuclear region of nonciliated cells is an often large collection of lysosomes (Figs. 5a & 6a). These supranuclear lysosomes are also found in the ciliated cells of the



rat oviduct (Fig. 6b&c) and may eventually be involved in the formation of giant autophagic lysosomes (Figs. 6c, 7 & 21a-GAL).

The rabbit oviduct also contains granulated and nongranulated nonciliated cells. The nongranulated type of cell is found predominantly in the ovarian end of the ampulla (Fig. 8-NC). The nonciliated cells of the rest of the ampulla and the isthmus are mainly granulated. The secretory granules, similar to those of the rat, vary in density and size. Some may be extremely electron-dense while others are larger and more electron-lucent, although containing denser patches (Figs. 10a & 11b). It has been suggested that both the rough endoplasmic reticulum and the Golgi apparatus contribute to the formation of secretory granules and the ultrastructure of the oviductal granulated cells is consistent with this suggestion (Figs. 9a & 10a). In the isthmus in particular the entire volume of the nonciliated cells appears to be involved in granule formation (Fig. 9b). Production of secretion is probably a continuous process in the estrus rabbit, and as evidenced from the various profiles presented (Fig. 10a&b) the individual cells' secretory cycles do not appear to be synchronized.

Secretory granules are already present at three weeks in the rat and at eight weeks in the rabbit, although in the latter species the size and number of the granules are greatly increased in the older animals (Fig. 11a&b).

ii. Ciliated Cells. The other major cell type represented in the oviductal epithelium is the ciliated cell. Although exhibiting features common to those of other mammals, the ciliated cells of both the rabbit and rat oviducts contain features that seem to be peculiar to

their individual species. The basic cilium structure of nine peripheral microtubule doublets with a central pair of microtubules is common to both species, as is the arrangement of nine microtubule triplets in the basal body.

Rat cilia have attached to their basal bodies irregular root structures having either a pointed (Figs. 15b & 19b) or bulbous (Fig. 12c) shape. In addition, a cone-shaped structure exhibiting two or more periodic bands and known as a basal foot may be found attached to the lateral aspect of the ciliary basal bodies of the rat (Figs. 12b, 15b & 19b).

In the rabbit the only appendages to the cilia are a cluster of 'knob' like structures around the base of the basal bodies (Fig. 12a-k). An unusual organelle found in the cytoplasm of the rabbit ciliated cell is the glycogen body. This structure, originally described by LeBeux (1969), is found in the perinuclear cytoplasm and consists of an ovoid or spherical collection of concentric membranes with associated glycogen particles (Fig. 13a&b). Although LeBeux claimed that glycogen bodies were not present in immature animals, they were observed in the present study in rabbits of two months of age (Fig. 13a).

### iii. Mitochondrial Inclusions in Rat Oviductal Ciliated Cells.

Rat oviductal ciliated cells are unusual in that their mitochondria are often found to contain paracrystalline inclusions (Fig. 14). These inclusions are composed of parallel arrays of filaments measuring approximately 8nm in diameter. The length of the individual filaments vary, averaging from approximately 0.6 to 0.9  $\mu$ . However inclusions have been found that measure up to 8  $\mu$ .

The appearance of the inclusions changes with the plane of section.

In some longitudinal sections the filaments are regularly arranged whereas in others the center to center spacing varies (Fig. 14c). Cross sections of the inclusions reveal a hexagonal (Fig. 14d) or quadrilateral gridlike appearance. At high power each filament appears to be composed of a linear array of globular subunits.

The paracrystalline inclusions are located within the matrix of the mitochondria. Most of them fill the mitochondrion entirely, distorting its shape to varying degrees. Only a few cristae are identified within the mitochondria (Fig. 14c, arrows).

Mitochondria containing inclusions stain intensely with toluidine blue, which facilitates their location with the light microscope (Fig. 14b). At this level it is possible to determine to what extent the cells in any particular sample contain inclusions. Mitochondrial inclusions have not been detected at either the light or electron microscope level in any other oviductal cells apart from those bearing cilia. Cells containing inclusions are found predominantly in the preampulla and adjacent ampulla, which is not surprising considering the preponderance of ciliated elements in these regions of the oviduct. There is however, a large amount of variation in the number of ciliated cells containing inclusions in oviducts taken from different rats. On the whole, the oviducts of the majority of rats contained a few ciliated cells with mitochondrial inclusions, whereas only a few samples contained many such cells.

Paracrystalline arrays are never observed to be free in the cytoplasm, although certain tangential sections make the mitochondrial membrane difficult to distinguish (Fig. 14e). In addition, neither inclusions nor mitochondria containing inclusions are observed being extruded from the cell. And finally, mitochondrial inclusions

were never observed in the ciliated cells of the rabbit oviduct.

iv. Ciliogenesis. Ciliogenesis is observed in both the rat and rabbit oviducts. Ciliogenic cells are most common in the immature animals of each species but they are also found in the mature rat preampulla. The fimbriated end of the oviduct of the mature rabbit was not examined at the electron microscope level, and so no conclusions can be made concerning the occurrence of ciliogenesis in the older animals of this species.

The formation of cilia in mammalian ciliated cells has been analysed at the ultrastructural level by Dirksen and Crocker (1965), and it is from their work that the terminology used in the brief account that follows was obtained. As described by these authors for the fetal rat trachea and by Dirksen (1971) for the fetal mouse oviduct, ciliogenesis is initiated by the association of a mature centriole with structures known as proliferative elements. These structures (Fig. 15a&b-PE) are large masses of slightly electron-dense fibrillar material containing small dark granules. The granules are presumed to enlarge and detach themselves from the main mass, thus becoming condensation forms (Figs. 15 & 16b-CF). Developing centrioles appear around the condensation forms, and this association is known as a generative complex. The mature centrioles, or basal bodies, migrate to the cell surface and develop ciliary buds. In any ciliogenic cell examined, several stages in the process may be observed, thereby suggesting that the development of the cilia in an individual cell is asynchronous.

Ciliogenesis in the rabbit oviduct is essentially the same as that observed in the rat. The proliferative elements in this species

however do not resemble the fibro-granular complexes seen in the mouse and the rat. Instead, clusters of small dense granules, similar to those found within the rat proliferative element, accumulate in the apical region of the cell (Fig. 16a-PE). These cells in the initial stages of ciliogenesis are also distinguishable in the rabbit oviduct by the presence of very regular microvilli measuring approximately  $0.5\ \mu$  in length.

Typical condensation forms and generative complexes can be found in the rabbit ciliogenic cell (Fig. 16). As the developing centrioles proceed to the apical region of the cell to establish themselves as basal bodies they are often observed to be associated with small dark granules resembling proliferative elements. This association is also observed in the rat oviduct. In both species the ciliary buds forming from the new basal bodies are often found developing into invaginations of the apical membrane, or vacuoles beneath the cell surface, which later open on to the lumen (Fig. 16b).

According to some authors (Brenner, 1969b; Biava and Matsuura, 1967) the proliferative elements, possibly through their formation from polysomes, may contain RNA capable of initiating the formation of centriolar subunits. Using a method described by Smith and Stuart (1971) ultrathin sections of rabbit oviduct were treated with unbuffered silver nitrate, which selectively stains ribosomal proteins. This was done to test the hypothesis that the proliferative elements originate from the fusion of polyribosomes.

Figure 17a shows a ciliogenic cell, and in the region of the developing basal bodies can be seen groups of polyribosomes which are stained by the silver. Four proliferative elements are visible in this section and it is obvious that they are not stained as

intensely as the neighbouring polyribosomes. At higher power (Fig. 17b) the absence of distinctive silver staining on the proliferative elements is clearer. However at this level a faint background staining is apparent that can also be seen on the developing basal bodies. The significance of this background staining is not known.

v. Ciliary Vacuoles. Ciliary vacuoles, the subject of much controversy in the literature, are observed in both the rat and rabbit oviducts (Figs. 18, 19 & 20-CV). These structures are large intracellular vesicles with a varying number of cilia projecting into them. Within the cell the vacuoles are usually located close to the basal lamina. In fact, many of the cells containing ciliary vacuoles appear to be 'basal' cells (Fig. 18), that is they do not appear to reach the oviductal lumen. They have however been observed within typical columnar cells, which do not bear cilia on their luminal surface (Fig. 20c).

The most frequent form taken by cells containing ciliary vacuoles in the oviducts of both species is seen in Figure 18. The oval-shaped vesicle seems to be aligned with its long axis parallel to the basal lamina. The nucleus of the cell assumes a lateral position and the vesicle appears to indent it, giving it a half-moon shape. Projecting into the lumen of the vacuole are microvilli and a varying number of cilia. Occasionally the vacuoles are tightly packed with cilia (Fig. 19b) but there are usually far fewer cilia than that found on the luminal surface of ciliated cells. A varying amount of debris occupies the intravesicular space between the cilia and microvilli. The cytoplasm surrounding the vesicle often contains fibrils which may serve as support (Figs. 18b & 20a-f).

The mechanism of formation of ciliary vacuoles is somewhat obscure. In some instances (Fig. 18a) the vesicle itself seems to be formed by the fusion of segments of rough endoplasmic reticulum which have been distended by an electron-lucent material. Upon fusion, the ribosomes are lost from the membranes and the cytoplasm surrounding the growing vesicle produces cilia and microvilli which project into the interior of the vesicle. Lysosomes do not appear to be directly involved in the formation of the vacuole, although bits of debris found within the vacuole may exhibit acid phosphatase activity (Fig. 20b).

The process of ciliogenesis observed in association with ciliary vacuole formation appears to differ from that observed usually in the ciliated cells of the oviductal epithelium. Centriole replication and subsequent cilia formation seem to occur in the absence of any of the fibro-granular precursors previously described (see iv. Ciliogenesis). Centrioles become associated with a membranous vesicle or vesicles, known as primary ciliary vesicles, and a ciliary bud begins to invaginate into the vesicle, thus acquiring its covering membrane (Fig. 18b & 19b-PV). In several cells, clusters of centrioles were found in the cytoplasm in close proximity to groups of membranous vesicles (Fig. 19a & 20c). Eventual coalescence of the primary ciliary vesicles with associated ciliary buds would in these cases presumably give rise to the single large ciliary vacuole. However in other cases the ciliary buds appeared to grow directly into the ciliary vacuole, and in one instance a ciliary bud appeared to be forming in a distended segment of rough endoplasmic reticulum (Fig. 18a-arrow).

Although ciliogenesis involving fibrogranular precursors has not been observed in association with ciliary vacuole formation, prolifer-

erative elements were seen in the basal cytoplasm of one cell (Fig. 21a-PE). Whether the unusual location of these proliferative elements meant that they would subsequently be involved in ciliary vacuole formation can not be answered.

The eventual fate of ciliary vacuoles is also obscure. The general consensus of previous authors is that the vacuoles migrate to the surface of the cell and fuse with the apical cell membrane, allowing the cilia and microvilli to face the oviductal lumen. The vacuole in Figure 20a might be in the process of migrating upward; the nucleus of the cell has assumed a basal position and the vesicle seems to be pushing the nucleus of the cell immediately superior to one side. However, actual fusion of a ciliary vacuole with the surface membrane, with concomitant release of intravesicular debris and extraversion of the cilia and microvilli into the oviductal lumen, was never observed.

vi. 'Transitional' Epithelial Cells, Degenerating Cells and 'Wandering' Cells. Transition of ciliated cells into secretory cells and vice versa is another controversial issue in the oviductal literature. In the present investigation a few examples of transitional cell types were observed in the mature rabbit oviduct, where several ciliated cells also contained typical secretory granules (Fig. 22). However these cells appeared to be only isolated cases among the majority of well-differentiated cells.

Although mitoses were never observed in the adult oviducts, both secretory and ciliated cells were occasionally observed to undergo degeneration. When this occurs the cytoplasm darkens and the cell is flattened by its neighbours (Fig. 23-DC). The remains of



these effete cells are most likely phagocytosed by adjacent epithelial cells and digested in giant isocytophagic lysosomes (see Appendix).

The columnar cells of the oviductal epithelium are joined together by typical apical junctional complexes (Fig. 12a) and to varying degrees, the lateral surfaces of neighbouring cells are connected by desmosomes. The epithelium rests on a thin continuous basal lamina. In the rabbit this membrane is very regular (Fig. 24b) but in the rat the basal lamina projects into the base of the cell at intervals (Fig. 24a). Oblique sections of these projections give the impression of segments of basal lamina isolated within the cytoplasm.

Desmosomes are less frequently found joining adjacent cells in the basal one-half to two-thirds of the epithelium. It is in this region of the intercellular space that the various 'wandering' cells, referred to as 'basal' cells by some authors, of the oviductal epithelium are found.

The structure of these cells identify them as lymphocytes and macrophages. The lymphocytes (Fig. 21-Lym) have a high nucleocytoplasmic ratio, and the thin rim of cytoplasm surrounding the dark nucleus with condensed chromatin contains abundant free ribosomes, but usually little rough endoplasmic reticulum. Pseudopodia projecting into the intercellular space between neighbouring epithelial cells are the chief hallmarks of the macrophages, along with varying amounts of rough and smooth endoplasmic reticulum and granules of a lysosomal nature (Fig. 25-M). There are no desmosomes connecting these wandering cells to their epithelial neighbours.

The only 'basal' epithelial cells observed in the rat and rabbit oviducts are those previously described containing ciliary vacuoles.

#### MORPHOLOGY OF THE RAT AND RABBIT OVIDUCTAL EPITHELIUM: DISCUSSION

Several aspects of the ultrastructure of the rat and rabbit oviductal epithelium have been covered previously (Bajpai et al., 1974; Borell et al., 1956 & 1959; Brower and Anderson, 1969; Hashimoto et al., 1959a&b; Jirsová, 1972; Jirsová and Kraus, 1974; Jirsová, Kraus and Martinek, 1971; Nilsson, 1957 & 1958b; Nilsson and Rutberg, 1960; and Shipstone et al., 1974). The preceding description reviews the basic fine structure of the various cell types found in the oviduct, and focuses on ultrastructural features that have not been dealt with in detail before.

Familiarity with the cellular architecture of the oviductal epithelium in the normal physiological state of the animal is essential for the detection of any alterations which may occur following experimental treatments. However it is often not possible to discern all of the functions of a cell from its ultrastructure. Therefore in this discussion an attempt has been made to supplement the results of the present electron microscopic study with biochemical, physiological, histochemical, autoradiographical and other evidence in order to come to the fullest understanding of the function of the oviductal epithelial cells. As this report also deals with some of the effects of estradiol on the oviductal epithelium, experimental evidence pertinent to the physiological regulation of oviductal function by estradiol will also be included.

### Nonciliated Cells and the Secretory Functions of the Oviduct

In both the rat and rabbit oviducts a variety of nonciliated cells are found. Because some of these cells contain granules a secretory function has been presumed. A sulphated mucopolysaccharide composition of the secretion is suggested by the fact that the oviductal epithelial cells of these species are observed to incorporate radioactive sulphur, especially in those cells containing granules (Boström and Odeblad, 1952). An acidic glycoprotein containing sulphated sugars has been isolated from rabbit oviductal fluid and, using a fluorescent antibody technique, traced to the mucin layer of three-day-old rabbit eggs (Shapiro, Brown and Yard, 1974). The importance of estradiol in promoting secretion formation is indicated by the fact that uptake of isotopic sulphur is greatest in the rabbit during estrus and following the administration of the hormone (Yasuda, 1966; Zachariae, 1958).

The regulation of oviductal secretions by estrogen has been extensively examined in the rabbit (Bishop, 1957; Greenwald, 1958 & 1969; Mastroianni et al., 1961). Although estrogen alone will restore the volume of oviductal fluid in the rabbit to preovariectomy levels, progesterone is necessary for the release of the mucin granules found in the isthmus and adjacent part of the ampulla (Greenwald, 1958). Brower and Anderson (1969) confirmed with the electron microscope that although formation of secretory granules occurs at all times in this species, release of the granules is greatest during egg transport, i.e. when the level of plasma progesterone is increasing.

Because of the small size of the rat it has as yet not been possible to examine the composition and biochemical properties of the oviductal fluid of this species. Nor has the hormonal control of oviductal fluid volume or secretory granule formation and release been determined. Deane (1952) found no differences in the amount of PAS positive material present in the isthmic cells of the rat oviduct at the different stages of the estrus cycle. Although Reinius (1970) detected fewer secretory and glycogen granules in the nonciliated cells of the mouse oviduct (which closely resembles that of the rat) in those segments containing the eggs or zygotes, active release of secretory products was not reported in the electron microscopic examination. However the apical position of the granules in the mammalian oviducts coupled with the similar staining properties of the luminal contents imply that the oviductal 'secretory' granules contribute to the formation of the oviductal fluid.

The bulk of the oviductal fluid arises by transudation from the vascular and/or lymphatic systems (Marcus and Saravis, 1965). That this process is an active, rather than a passive transfer, is indicated by the fact that the concentrations of the constituents of serum and oviductal fluid differ, with (e.g. in the rabbit) the level of protein being lower in the oviductal fluid than in the serum, whereas the concentrations of bicarbonate and amylase are higher (Feigelson and Kay, 1972; Hamner and Fox, 1969). In addition to the granulated nonciliated cells found in the rat and rabbit oviducts there are also several nongranulated nonciliated cells, whose function cannot be determined from their ultrastructure. It is possible that these

cells might be involved in the transfer process.

The passage of substances through the oviductal epithelium to the egg or embryo has been visualized at the light microscope level in the mouse (Glass, 1969; Glass and McClure, 1965). Using fluorescent antibody techniques it was found that the transfer of serum macromolecules to the egg occurred mainly in the region of the ampulla and that all cell types of the epithelium participated. Further studies of the transfer process suggested that passage of blood macromolecules into the oviductal epithelium is governed by estrogen levels. It could not be determined, however, at what stage in the transfer sequence estrogen exerted its effect.

The ability of the human oviductal epithelium to transfer isotopic serum albumin has been examined in *in vitro* conditions (Lefebvre et al., 1972). It was found that the albumin could pass through the epithelium in either direction, and again, the process seemed to be influenced by the hormonal status of the oviduct.

Transfer of macromolecules from the vascular system to the oviductal lumen through the epithelial cells has yet to be specifically traced in the rabbit or rat, however the presence of serum proteins in the oviductal fluid of the former species suggests that such a transfer does occur. Using horseradish peroxidase and ferritin as tracers, Schlafke and Enders (1973) studied the ultrastructure of protein uptake by rat pre-implantation stages and found that two-cell embryos contained small amounts of the tracers in micropinocytotic vesicles. A combination of either of the protein labels with suitable *in vitro* techniques might clarify the presence of a macromolecular transfer system in the rat and rabbit oviducts, and, in addition,

reveal which epithelial cells were involved.

There is also evidence available that the nonciliated cells of the oviduct may participate in a local secretory immune system (to be discussed later). Using immunofluorescent techniques, a secretory component, believed to be synthesized by the Golgi apparatus, has been observed in the columnar epithelium of the human oviduct (Tourville et al., 1970). This is further evidence that until suitable cytochemical or labeling techniques are applied, conventional ultrastructural examination of the oviduct will not reveal all of the secretory functions of the epithelial cells.

#### Ciliated Cells and Ciliogenesis

The ciliary apparatus of the rat and rabbit oviductal cells does not differ markedly from that described for other species. The amount of cyclic change observed in the ciliated cells of both these species, and indeed in several others, is the subject of much differing opinion (see Brenner, 1969a for review). In the present investigation deciliation and regrowth of new ciliated cells was not observed on a large scale during the estrus cycle of the rat. Isolated cells involved in ciliogenesis, however, were seen in most samples of mature rat oviduct studied, particularly in the preampullar region, and this is in accord with previous studies (Jirsova, 1972).

No instances of ciliogenesis were observed in the mature rabbit oviduct, which is in agreement with the work of McCarron and Anderson (1973). In a study of the postnatal development of the oviductal epithelium they found no ciliogenic cells beyond twenty days of age. However in the light of the fact that no examination of the fimbriated part of the oviduct, which consists mainly of ciliated cells, was made

in either their study or the present one, the possibility of ciliogenesis occurring in the mature rabbit oviduct cannot be ruled out.

The process of ciliogenesis in the mammalian oviduct becomes visible at the ultrastructural level when tubulin (i.e. microtubule protein) from cytoplasmic pools of the protein dimer is aggregated into various centriole precursors (Staprans and Dirksen, 1974).

The first of these precursors to be observed is the proliferative element, which in the mouse and rat oviducts, consists of a mass of fibrillar material with associated electron-dense particles. Large electron-dense masses known as condensation forms become associated with developing centrioles in generative complexes. Fully formed centrioles migrate from these complexes to the apical surface of the cell and become the basal bodies of developing cilia. In an extensive study of this process in the developing mouse oviduct, Dirksen (1971) found morphological evidence suggesting that the condensation forms were the source of microtubule protein (i.e. tubulin) for the growing centrioles in the generative complex. However recent biochemical studies (Dirksen and Staprans, 1975; Staprans and Dirksen, 1974) have not been able to prove that tubulin from the cytoplasmic pool passes through the centriolar precursor structures prior to incorporation into the nascent centrioles and the ciliary axonemes.

The events described above appear to occur during ciliogenesis in the oviducts of all of the mammalian species so far examined (Brenner, 1969a&b; Dirksen, 1971; Jirsová, 1972; McCarron and Anderson, 1972; and Verhage et al., 1973b). The observations in this study concerning the morphology of ciliogenesis in the developing and mature rat oviduct indicate that the process is identical to that observed during differentiation of the respiratory epithelium in the rat

trachea (Dirksen and Crocker, 1965) and during postnatal development of the mouse oviduct (Dirksen, 1971).

However the ultrastructure of the proliferative event in the above species differs from that described in this report for the rabbit oviduct. Although designated as proliferative elements, the small electron-dense granules present during the initial stages of ciliogenesis are not aggregated into the fibrogranular masses which are so characteristic of the process in the mouse and rat. Also peculiar to the rabbit oviductal ciliogenic cell is the presence of very regular surface microvilli. These microvilli are typical of cells in the early stages of ciliogenesis when the cytoplasm primarily contains proliferative elements.

Similar clusters of electron-dense proliferative elements are found in ciliogenic cells of the rhesus monkey oviduct (Brenner, 1969a&b). Brenner observed that the proliferative elements characteristic of the initial stages of ciliogenesis were also seen in association with newly established basal bodies and he believed them to be involved in rootlet formation (1969b). Cilia in the rabbit oviduct do not possess rootlets, however McCarron and Anderson (1972) suggested that the association of the proliferative elements with the newly formed basal bodies possibly implicated them in the formation of the knob-like appendages attached to the basal bodies of rabbit oviductal cilia.

The composition of the proliferative elements is not precisely known. Dirksen and her colleagues believe that the condensation forms are derived from the electron-dense granules of the fibrogranular mass, thus implying that the granules are aggregates of tubulin. Their belief is that once the microtubule protein is



synthesized, no other mediator is necessary for its assembly into the centriolar and axonemal structures. However if this is true, then the association of proliferative elements with the rootlets and knoblike structures of basal bodies must be fortuitous as the morphology of these appendages does not indicate a microtubular structure. Alternatively the adjacent proliferative elements could be providing the newly formed basal bodies with tubulin for ciliary axonemal construction.

In a report of ciliogenesis in regenerating adult rat tracheal epithelium, Biava and Matsuura (1967) claimed that the proliferative elements originated from a transformation of polyribosomes (polysomes). Dirksen and Crocker (1965) however, made no mention of this in their description of ciliogenesis in the fetal rat trachea. In the present study a method used to stain the proteins associated with ribonucleic acid revealed that the staining of proliferative elements and adjacent polysomes was sufficiently dissimilar to discount the transformation theory.

In this method (Smith and Stuart, 1971) silver ions bind to the protein component of nucleic acid-protein complexes. Polysomes of ciliogenic cells (and also of the other oviductal cells) are stained with several silver particles of somewhat varying sizes. The proliferative elements however display only very faint silver particles which tend to be smaller than those observed on ribosomes. This faint staining is also seen on the microtubules of the basal bodies.

The reason for this faint staining of the proliferative elements and the basal bodies is unclear. Other procentriolar structures were not observed in silver-stained preparations and therefore it is not known whether condensation forms and generative complexes

also exhibit such staining. There is evidence that nucleic acid is associated with centrioles and that this nucleic acid may have some role in the replication of these organelles (Dirksen and Crocker, 1965). Hartman and his colleagues (Hartman, Puma and Gurney, 1974) found evidence that the nucleic acid associated with the basal bodies of Tetrahymena pellicles was a stable RNA which was not mitochondrial, transfer or ribosomal RNA. They proposed that the basal bodies contained this single stranded RNA held in a rigid configuration by protein. If this is true, then the small silver particles observed here in the basal bodies could be associated with the proteins related to the basal body RNA. However this still does not explain the similar staining of the proliferative elements.

In the majority of species studied so far, growth of ciliated cells and ciliogenesis in the oviduct is under hormonal control (see Brenner, 1969a for review). Either by injecting the hormone into immature animals to induce ciliation of the epithelium or by hormonal replacement therapy of the ovariectomized animal, it has been found that estrogen is responsible for the development and maintenance of the ciliated cells in the rhesus monkey (Brenner, 1967), dog (Verhage et al., 1973b), cat (Verhage and Brenner, 1975), and rabbit oviducts (Rumery and Eddy, 1974).

In the normal human endometrium, ciliated cells and ciliogenesis are most commonly observed in the late proliferative stage. An increase in ciliated cell numbers is frequently encountered in cases of endometrial hyperplasia, a condition known to be associated with hyperestrogenism. This is further evidence implicating estrogen in the hormonal control of these cells (Masterton, Armstrong and More, 1975;

Schuessler, 1973).

The fimbriated end of the rhesus monkey's oviduct undergoes a deciliation and subsequent reciliation during each menstrual cycle. Brenner and his colleagues (Brenner and Resko, 1972; Brenner, Resko and West, 1974) have carefully evaluated this process, and their results have beautifully illustrated how the alteration in dominance of the two principle ovarian hormones can affect the behaviour of the target tissues. Using hormone therapy to establish artificial oviductal cycles in ovariectomized monkeys, they found that when the ratio of plasma progesterone to plasma estradiol was approximately fifty to one the fimbrial epithelium would lose its cilia. Ciliogenesis would occur when the plasma progesterone had dropped to such a level that the ratio between the two hormones (P/E) was ten to one (the level of estradiol remains fairly constant throughout both the natural and artificial cycles).

Not all mammals undergo a cyclic loss of cilia. It also appears that not all oviducts are under similar hormonal control mechanisms. Nyak and Zimmerman (1971) found that both estrogen and progesterone could restore the ciliated epithelium in the ovariectomized pig's oviduct. In a study of the hormonal control of the ciliated cells of the rat oviduct, Dubuisson and his colleagues (Dubuisson et al., 1972) came to the conclusion that in this species, for some reason, ciliated cell growth and ciliogenesis are autonomous processes. They found no loss of cilia from the oviducts of ovariectomized rats six months after the operation. They also found that neonatal ovariectomy had no effect on the subsequent differentiation of the epithelium; ciliated cells began to appear in the oviducts of all rats six days after birth, regardless of whether or not they had been operated on

the day of birth. In addition, estradiol administered to some of the rats ovariectomized at birth did not provoke an early appearance of ciliated cells. And finally, intrarenal grafts of oviducts from newborn rats developed in a similar manner, regardless of the sex of the recipient animal.

### Ciliary Vacuoles

Ciliary vacuoles in the mammalian oviductal epithelium were first described by Mihálik (1934). In a light microscopic study of the rabbit oviduct he found many of these intracellular vesicles containing cilia, and he named this structure the 'Flimmerblase'. Brenner (1969a) reviewed the literature concerning ciliogenesis and ciliary vacuoles and concluded that in the absence of positive evidence from ultrastructural studies, the 'Flimmerblase' must remain a mystery.

Structures closely resembling those described by Mihálik were observed in the present study in both the rabbit and rat oviducts. The ultrastructure of these vacuoles clearly show their intracellular nature. Close proximity to the basal lamina and uneven distribution of cilia along the vacuolar membrane are strong indications that these ciliary vacuoles are not merely indentations of the apical cell surface. The unsuitability of the indentation theory is further shown in those instances where the ciliary vacuoles occupy a middle position in a cell showing a luminal surface.

Rasweiler (1972) observed ciliary vacuoles in the bat oviduct, and through serial sections at the light microscope level showed that there was no continuity between them and the oviductal lumen. He believed that the ciliated vacuoles developed in spaces made in

the epithelium by autolysing eosinophilic leucocytes or mast cells which had migrated from the lamina propria. However no electron microscopy has been performed on the oviduct of the bat to confirm this theory.

The present ultrastructural examination suggests that there are two possible mechanisms for the formation of ciliary vacuoles. These involve either the fusion of distended segments of rough endoplasmic reticulum with the subsequent ingrowth of cilia, or else the coalescence of clusters of small vesicles, some of which act as primary ciliary vesicles in cilia formation.

Centriole replication and subsequent cilia formation can occur in several ways in living organisms (see Brenner, 1969a). Although ciliogenesis observed normally in the oviductal ciliated cells involves the fibrogranular precursor pathway of centriolar replication, this does not appear to be the case in ciliogenesis in ciliary vacuoles. The lack of proliferative elements, condensation forms and generative complexes in the cytoplasm surrounding the ciliary vacuole, suggests that the centrioles have arisen from repetitive self-duplication of the cell's diplosomal centrioles. Indeed the two methods of centriole formation may not be mutually exclusive; Dirksen (1971) has very occasionally observed procentrioles surrounding mature centrioles in a modified generative complex in mouse oviductal ciliogenic cells.

The association of centrioles with a primary ciliary vesicle (Sorokin, 1962) followed by subsequent shaft assembly has been observed in several instances of ciliary and flagellar development. Sorokin (1962) suggested that the membranous vesicles came from the Golgi complex, whereas in the water mould, Allomyces, the

vesicles came from pinocytosis of the cell membrane (Renaud and Swift, 1964). From their observations on ciliogenesis in the cells of the rat subcommissural organ, Lin and Chen (1969) have suggested that close proximity of a basal body (centriole) to a membrane is sufficient stimulus to induce ciliary shaft formation. This is supported in the present study by the observations of ciliary buds extending from centrioles into primary ciliary vesicles, large ciliary vacuoles, indentations of the apical cell membrane and even distended segments of rough endoplasmic reticulum.

It is difficult to suggest any possible reason for the presence of ciliary vacuoles in the oviductal epithelium. They have been observed in 'basal' epithelial cells and columnar nonciliated cells, but never in typical columnar ciliated cells, which may be the reason for the different mechanism of cilia formation. Under certain conditions nonciliated oviductal cells have also been observed to form large intracellular microvillous vesicles (see Parts II & III) in a manner analogous to that observed with ciliary vacuole formation. These 'intra-epithelial secretory cysts' seem to be caused by an upset in the hormonal stimulation of the oviduct. In light of the structural similarity of ciliary vacuoles to these microvillous vesicles, it seems likely that they represent a pathological condition of oviductal cells.

#### Mitochondrial Inclusions

One of the most unusual observations in this study was the finding of paracrystalline inclusions in the mitochondria of the rat oviductal ciliated cells. In a paper dealing with the mitochondrial variability in the rat oviduct, Jirsová and his colleagues

(Jirsová, Kraus and Martínek, 1973) mentioned the presence of osmiophilic lamellar inclusions in the mitochondria of some of the epithelial cells. Contrary to the evidence presented in this report, they stated that inclusions could be found in ciliated, nonciliated and 'reserve' cells, although the individual cell types could not be identified in the accompanying photographs. They found a correlation between the presence of mitochondrial inclusions and a paucity of rough endoplasmic reticulum and free ribosomes, with a concomitant increase in secondary lysosomes. A similar correlation could not, however, be found in the present study.

Several types of mitochondrial inclusions have been described in a variety of tissues and species (see Munn, 1974 and Suzuki and Mostofi, 1967 for reviews). In the rat they have been found in brain astrocytes (Mugnaini, 1964), cells of the thick limb of Henle in the kidney (Suzuki and Mostofi, 1967), thyroid follicular cells (Fujita and Machino, 1964; Nunez, Greif and Gershon, 1975), and cells of the placenta (Ollerich, 1968). Only the inclusions found in the placenta and thyroid cells bear a close morphological resemblance to the paracrystalline arrays of the rat oviductal mitochondria.

The presence of mitochondrial inclusions has been ascribed to several physiological and pathological conditions. Paracrystalline arrays closely resembling those found in the rat oviduct, thyroid and placenta have been observed in the mitochondria of liver cells of women during pregnancy or undergoing contraceptive therapy (González-Angulo et al., 1970; Martínez-Manautou et al., 1970). These authors suggested that the structure of the inclusions was similar to that seen with the crystallization of certain enzymes

observed *in vitro*, and therefore proposed that they represented abnormal quantities of enzymes. In another study (Themann and vonBassowitz, 1969- cited in Munn, 1974) high cytochrome oxidase and succinate dehydrogenase activities were found associated with the mitochondrial inclusions of human liver biopsy specimens.

The possibility exists that certain mitochondria might be capable of a limited amount of protein assembly, or even synthesis. This was suggested by Green and his colleagues (Green et al., 1971) in their review of mitochondrial structure and function. The main evidence for this proposal is circumstantial, and comes from Ward's observations (cited in Green et al., 1971) that the mitochondrial crystals of the frog oocyte have the same structure as the cytoplasmic yolk platelets. However, as the oviductal mitochondrial inclusions are never observed to be free in the cytoplasm, it does not seem likely that they are being synthesized for use within the ciliated cell (c.f. the yolk platelets).

One of the questions that arises from the observations made on the rat oviductal mitochondrial inclusions is why they are limited to the ciliated cells. Because of the globular nature of the subunits the individual filaments closely resemble the protofilaments of microtubules (Amos and Klug, 1974). In fact, the first impression received by inclusions that were several microns in length was that of 'intracellular' cilia.

Paracrystalline arrays of microtubule protein have been observed in the cytoplasm of anterior pituitary cells (Shiino and Rennels, 1974; Tseng and Kittlinger, 1974) and in cells treated with vinca alkaloids (Bensch and Malawista, 1969; Labrie et al., 1973). The schematic representation of three possible planes of section through



such crystals as illustrated by Bensch and Malawista (1969) closely resembles the varying profiles seen in sections of the oviductal inclusions.

It would be pleasant to be able to suggest that the inclusions found in the rat oviductal ciliated cells represented an abnormal crystalline accumulation of microtubule protein (i.e. tubulin) within the matrix of the mitochondria. One criticism of this hypothesis that immediately springs to mind is that this explanation would not account for the great similarity of these inclusions to those found in two other unciliated cell types of the rat, i.e. thyroid follicular cells and the placenta.

However Nunez and Gershon (1976) have recently described a ciliated stage in the development of the thyroid follicular cells of the dog. It was not clear what exactly happened to the cilia during the process of differentiation, for no broken or discarded ciliary shafts could be found within the follicles. One possibility was the resorption of the cilia and solubilization of the microtubules.

Ciliated thyroid follicular cells have also been observed in certain strains of mice (Ibid.) and if a similar step occurred in the differentiation of the rat thyroid follicular cells it is possible that the microtubule protein absorbed from the redundant cilia during development might somehow become sequestered within the mitochondria. This is pure speculation however and until further observations can be made, no conclusions as to the source or significance of the mitochondrial inclusions in the oviductal ciliated cells of the rat can be drawn.

### Oviductal Epithelial Differentiation and the Transformation Theory

Several theories exist to explain the origin of the differentiated elements of the oviductal epithelium. One theory, known as the transformation theory, proposes that ciliated and secretory cells represent different stages in the life cycle of a single oviductal cell (see Martínek, Kraus and Jirsová, 1967). Other researchers believe that the two cell types represent independent lines of development (Fredricsson, 1959) whereas a third theory suggests that both cell types arise from a basal 'reserve' epithelial cell (Pauerstein and Woodruff, 1967).

The possible transformation of ciliated cells into secretory cells and vice versa during the estrus cycle has been discussed by many authors. However insufficient evidence has been produced to either prove or disprove the theory. Fredricsson (1959) claims that following estrogen stimulation of the ovariectomized rabbit's oviduct, the secretory cells of the epithelium regenerate independently of the ciliated ones. However, ultrastructural examinations of the oviductal epithelium of the rabbit quite commonly reveal a few cells which possess both cilia and characteristic secretory granules (this report; Bajpai et al., 1974; Merchant, 1969; Shipstone et al., 1974). If ciliated and secretory cells were just different stages in the life cycle of a single cell type, one would expect to find many more cells in a transitional stage than are actually observed. The scarcity of such cells is more likely to be evidence in favour of a theory of a common origin for the two cell types.

Some clues as to the origin of 'mixed-phenotype' epithelial cells may be derived from studies of other heterogeneous epithelial populations. The origin, differentiation and renewal of the four

main epithelial cell types found in the mouse small intestine have been extensively investigated by Cheng and Leblond (1974). They found that all four cell types (i.e. Paneth, enteroendocrine, goblet and villus columnar cells) arose from a single stem cell type (crypt base columnar cells). The Paneth, enteroendocrine and goblet cells each contain secretory granules that are ultrastructurally quite distinct. Cheng and Leblond occasionally observed epithelial cells with two types of secretory granules, and they concluded that although the appearance of a mixed-phenotype implied some defect in the differentiation process, the presence of these cells was additional evidence for their common origin.

Evidence has been presented in the Introduction to this report that estrogen probably plays some role in the cytodifferentiation of the oviductal epithelium ( in certain species). The complexities of the differentiation process and its regulation will be discussed in more detail in Parts II and III of this report. However some observations made by Palmiter and Wrenn (1971) on tubular gland cytodifferentiation in the chick oviduct may be relevant to the problem of the origin of cells with mixed-phenotypes in hormonally regulated tissues.

Estrogen given to the immature chick will stimulate the thin oviductal epithelium to differentiate into tubular gland, goblet and ciliated cells. An initial proliferation of cells destined to become tubular glands is crucial to this process. Progesterone given concurrently with the estrogen will block this essential proliferation with the result that no glands develop. However electron microscopic examination of the oviduct reveals the presence of cells containing small amounts of granules, which Palmiter and

Wrenn called 'protodifferentiated' tubular gland cells.

They also observed that following three to five days of estrogen plus progesterone treatment, the chick oviductal epithelium contained several granulated cells involved in ciliogenesis. This suggested to them the transformation of the 'protodifferentiated' tubular gland cells into ciliated cells. It also could be taken as evidence for a common origin for these two cell types.

As Palmiter and Wrenn did not observe mixed ciliated and secretory cells in the oviducts of chicks treated with estrogen alone, and as mixed cells are only rarely observed in the oviduct of the estrus rabbit (this report) it would appear that progesterone might be interfering with the regulation by estrogen of the expression of cellular phenotype. Merchant (1969) and Shipstone and his colleagues (Bajpai et al., 1974; Shipstone et al., 1974) found that mixed ciliated and secretory cells were commonest in the rabbit oviduct 14-72 hours after mating. During this time the output of progesterone from the rabbit ovary is beginning to rise (Hilliard and Eaton, 1971).

Until further studies can be carried out, the exact cause of mixed ciliated and secretory cells cannot be determined. The relative scarcity of such cells however seems to give more support to the theory that ciliated and secretory cells arise from a common stem cell and that mixed cells represent a fault in differentiation, than to the theory that the two cell types are both phases in the life cycle of a single oviductal epithelial cell.

#### 'Basal' Cells in the Oviductal Epithelium

Pauerstein and Woodruff (1967) believe that it is a 'basal'

epithelial cell that gives rise to the ciliated and secretory elements of the oviductal epithelium. Their theory is based on evidence obtained from fluorescent and autoradiographic studies of the human oviduct performed at the light microscope level. Although their monographs (Woodruff and Pauerstein, 1969; Pauerstein, 1974) contain electron micrographs of the ciliated and secretory cells of the human oviduct, they present none of the basal cells, which they refer to as 'reserve' or 'indifferent' cells.

Other research groups have examined the ultrastructure of the 'basal' elements of the human oviductal epithelium, and identified the majority of these cells as lymphocytes and macrophages (Bullon, Bullon and Gonzalez, 1974; Jirsová, Martínek and Kraus, 1968). Although Bullon et al. describe basal epithelial cells, the feature that seems to distinguish these cells from the surrounding columnar cells is their intermediate height. Because of the thinness of section required for electron microscopy the appearance of basal epithelial cells in electron micrographs could result from normal columnar cells being sectioned obliquely. As seen in Figure 21a of this report, the oblique plane of section has given the epithelium a stratified appearance, but apart from the one obvious lymphocyte, all of the cells possess cytoplasmic and nuclear characteristics typical of the mature oviductal epithelial cells. The only epithelial cells observed in the rat and rabbit oviducts that appear to be truly 'basal' in position are those that contain ciliary vacuoles. No such vacuoles have been observed in the 'basal' epithelial cells of the human oviduct (Bullon, Bullon and Gonzalez, 1974).

Although describing basal epithelial cells, Bullon et al. clearly point out that it is the lymphocytes in the human oviductal

epithelium that have been classified as 'reserve' cells by Pauerstein and Woodruff. Odor (1974) has recently examined the question of 'basal' cells in the oviductal and endocervical epithelium of rabbits. Using ultrastructural criteria she also identified these cells as lymphocytes. To test Pauerstein and Woodruff's hypothesis (1967) that these cells give rise to the columnar cells of the oviductal epithelium she traced the estrogen-stimulated regeneration of the ovariectomized rabbit oviduct. All of the mitotic figures that she detected with the electron microscope were found in secretory cells, and the 'basal' cells were never observed undergoing mitosis. (Note: Mitoses never seem to be observed in ciliated cells-maybe because cells lose their cilia before undergoing mitosis-see McCarron and Anderson, 1973.) The ultrastructural characteristics of the lymphocytes and macrophages as described by Bullon et al. and Odor are the same as those of the cells observed in the rat and rabbit oviducts in the present study.

Bullon and his colleagues proposed that the lymphocytes and macrophages residing within the oviductal epithelium formed the basis for a local immunological defense mechanism. Local defense systems involving both cell-(Waldman and Ganguly, 1975) and humoral-(Vaerman and Ferín, 1975) mediated immunity have been postulated for the female reproductive tract. Representing a first line of defense against potential external pathogens, these local immunological systems would not necessarily involve the general lymphatic and blood circulation.

There is some experimental evidence that the oviductal epithelium is capable of mounting a local humoral-mediated response (Vaerman and Ferín, 1975). This mechanism would involve the production of

IgA by resident plasma cells within the oviductal stroma. The immunoglobulin would then be coupled with a secretory component synthesized by cells of the epithelium. The resulting secretory IgA would be released by the cells and retained in the apical mucus coating of the epithelium.

Using immunohistological techniques, secretory component has been localized in the epithelium of the human oviduct, endometrium and cervix (Tourville et al., 1970). The relative lack of IgA containing plasma cells detected in the oviductal and endometrial stroma when compared to that of the cervix was related to the fact that the environment of the former two tissues is usually sterile (Vaerman and Ferín, 1975).

Whether the lymphocytes and macrophages observed in the rat and rabbit oviducts participate in local immunological defense mechanisms has yet to be proven. However Pauerstein and Woodruff (1967) did notice an increase in the number of 'indifferent' cells in cases of chronic salpingitis of the human oviduct. And in the present study (see Part III), large numbers of small lymphocytes and cells believed to be macrophages were observed in the oviductal epithelium of a rabbit whose ciliated cells contained viral particles. All of the present morphological and experimental evidence suggests therefore that the small 'basal' cells found in the intercellular spaces of the oviductal epithelium are cells involved in immunological defense mechanisms, whether of a local or systemic nature, and not 'reserve' elements intended for the subsequent replacement of either the ciliated or secretory oviductal cells.

FIGURES 1-25

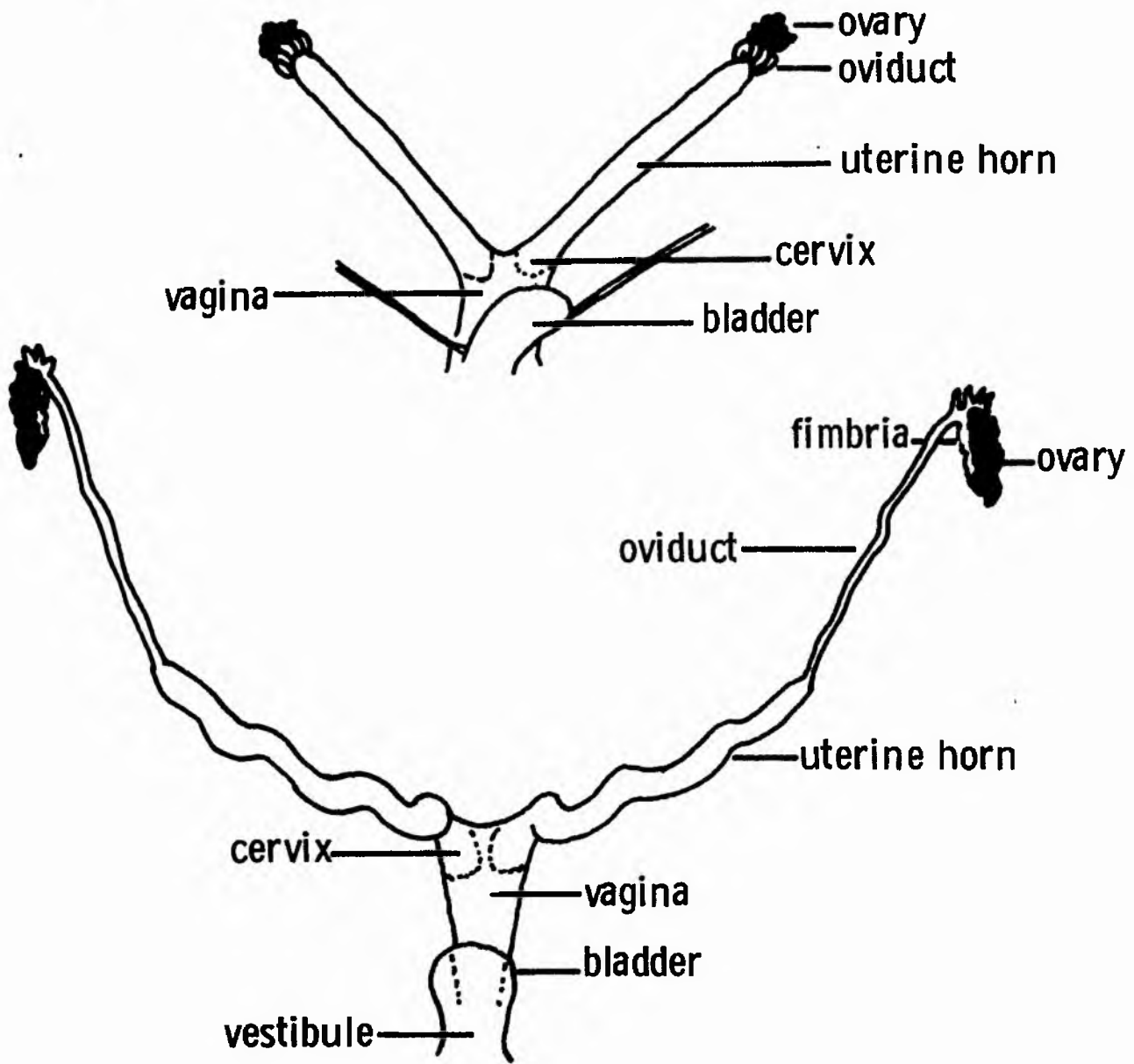


Figure 1: Diagrammatic representation of the female reproductive tract.

a. Rat.

b. Rabbit.

a.



b.

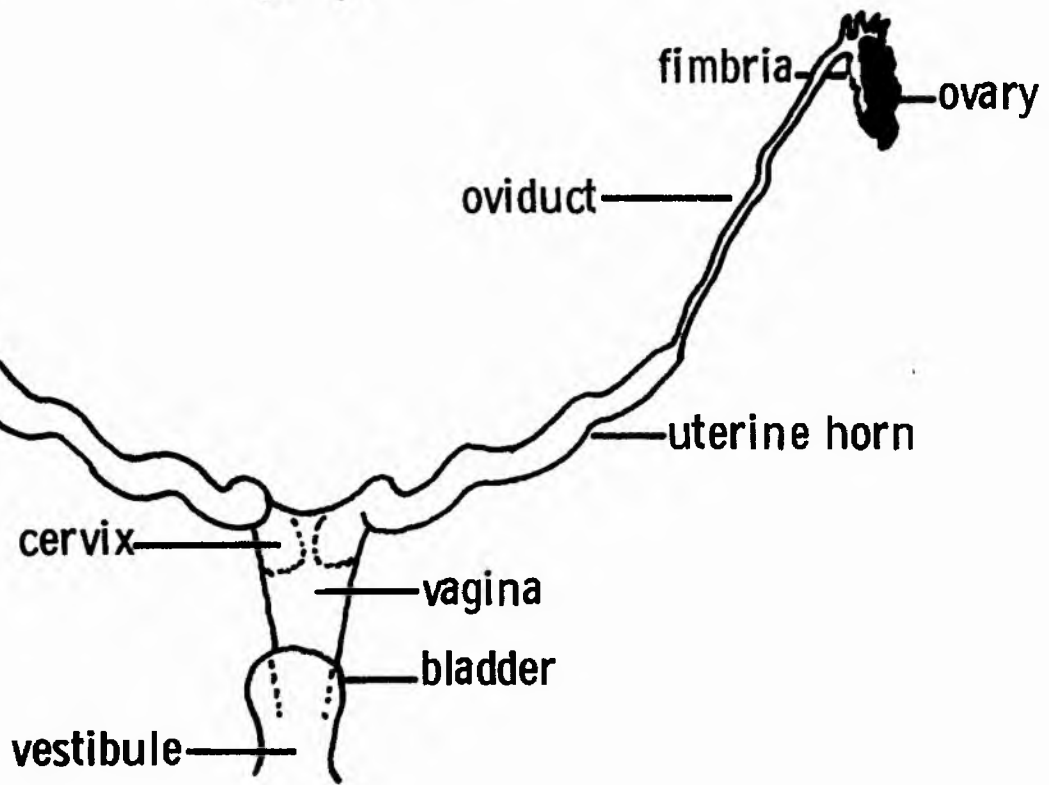


Figure 2: Rat Oviduct.

- a. Paraffin section of entire oviduct. H&E. X32

Note: Ovarian bursa has been detached.

a- ampulla

e- eggs and cumulus cells

f- fimbria

i- isthmus

j- junctura

p- preampulla

- b. Distended segment containing egg with cumulus cells  
(ampulla). H&E. X160

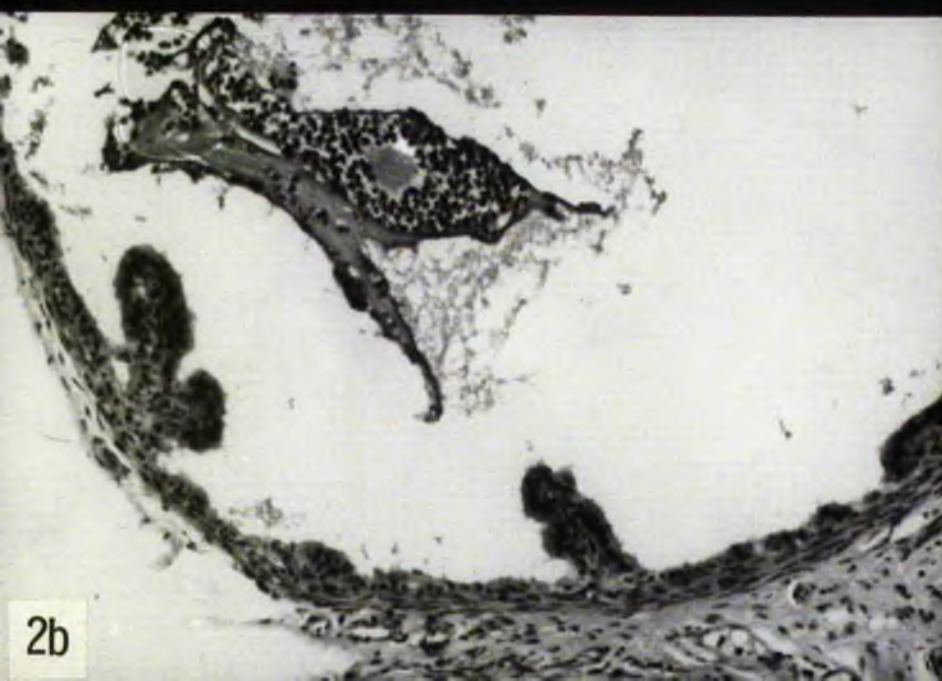
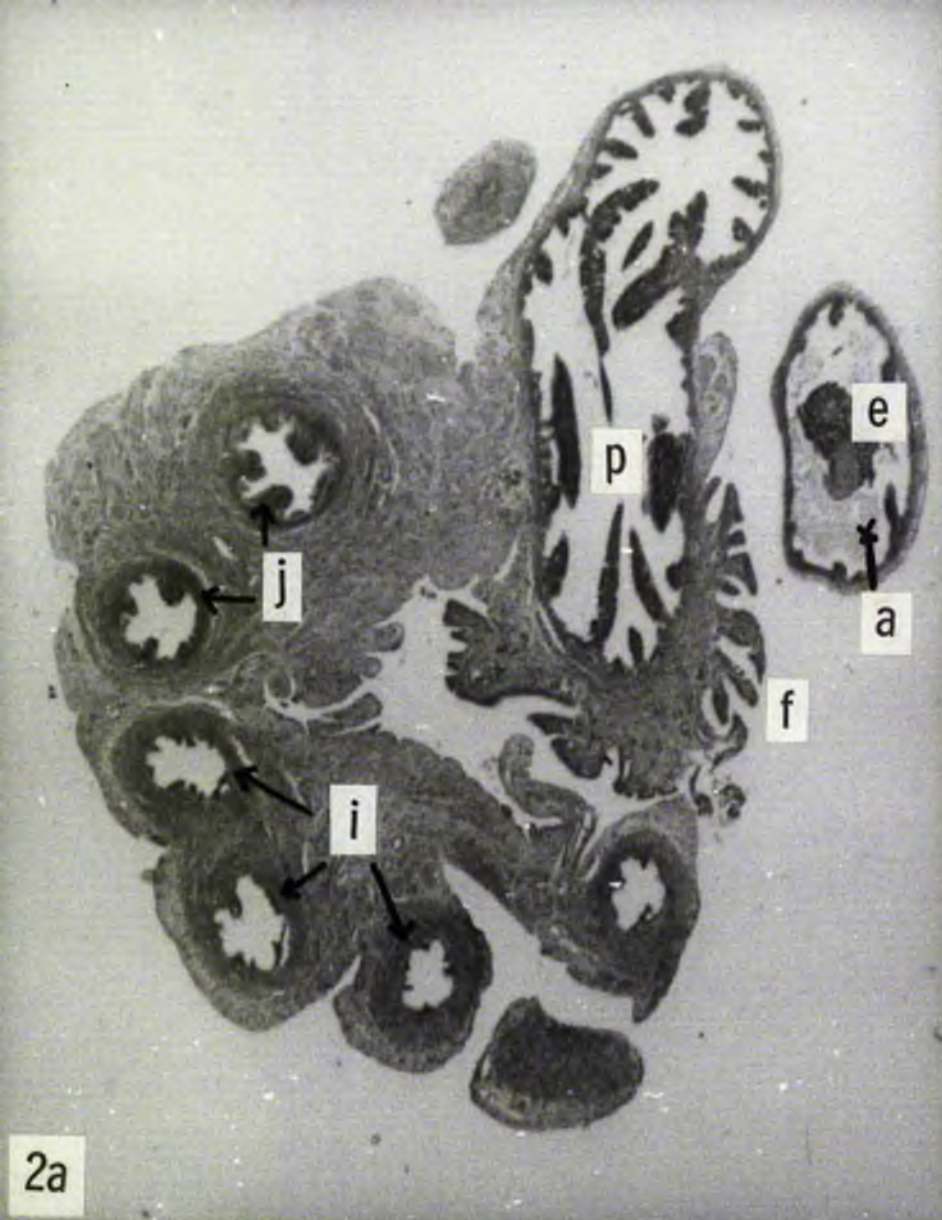


Figure 2 (continued):

c. 'Gland-like' configuration of cells on ampullar  
oviductal folds. H&E. X400

d. Isthmus. Toluidine Blue. X600

Note: Fixation of this sample was by aortic  
perfusion prior to immersion.

c- ciliated cell

e. Juntura. H&E. X160



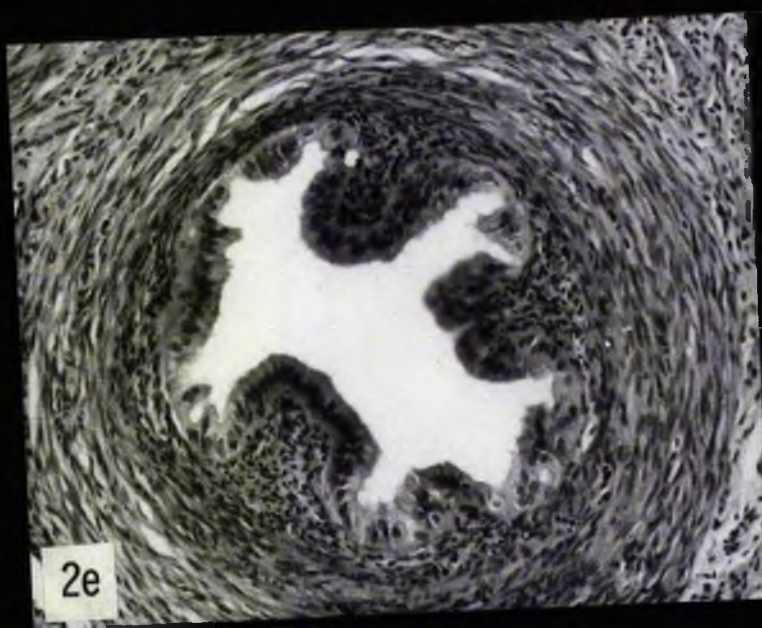
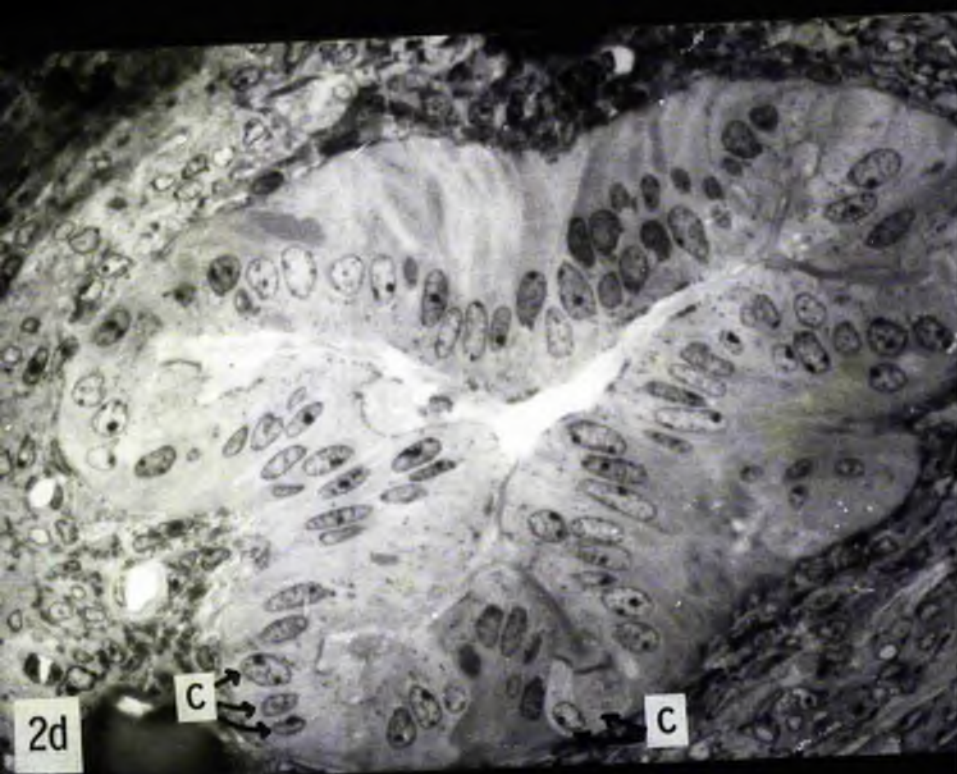


Figure 3: Rabbit oviduct.

a. Ampulla. H&E. X64

b. Isthmus. H&E. X64

c. Section of oviduct showing epithelial pit  
resembling sub-epithelial gland. Toluidine Blue.  
X200

L- oviductal lumen

P- epithelial pit







Figure 4: Isthmus of rat oviduct during proestrus. UA&LC. X3710

Note the ciliated cells in the crypt between nonciliated cells and the 'basal' epithelial cell containing a small microvillous vesicle (or possibly a future ciliary vacuole).

MV- microvillous vesicle



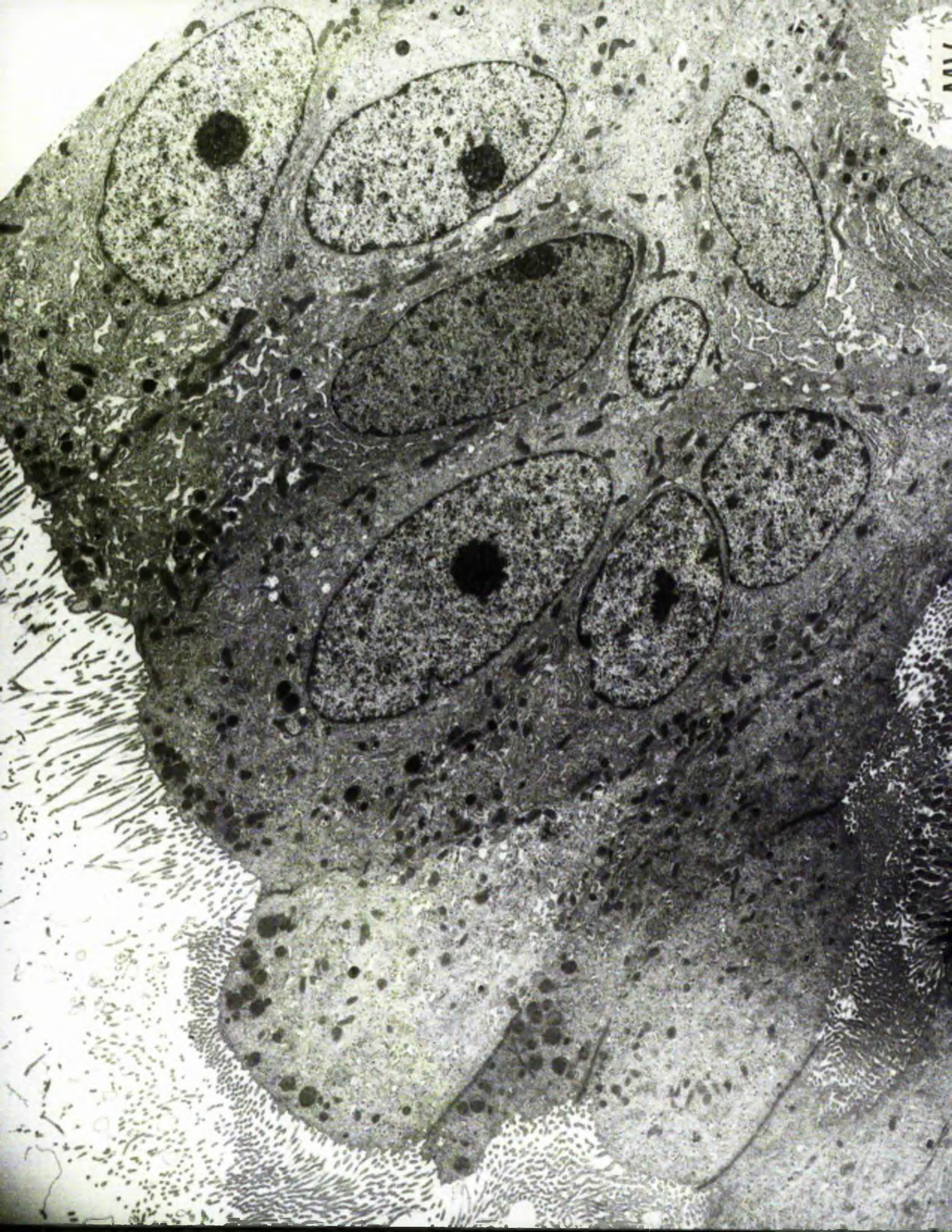




Figure 5: Rat secretory cells.

a. Isthmus during proestrus. UA&LC. X3710

SG- secretory granules

Ly- lysosomes

b. High power of secretory granules. UA&LC. X27,615

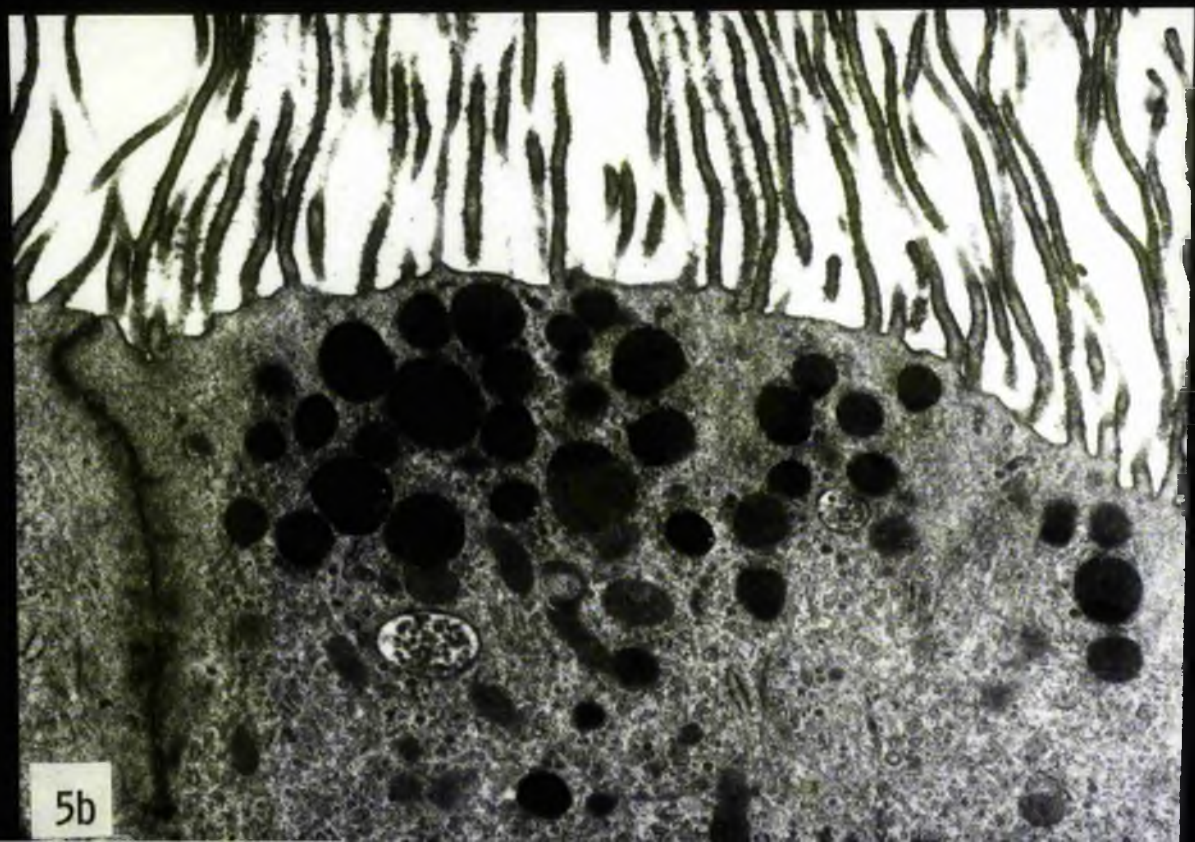
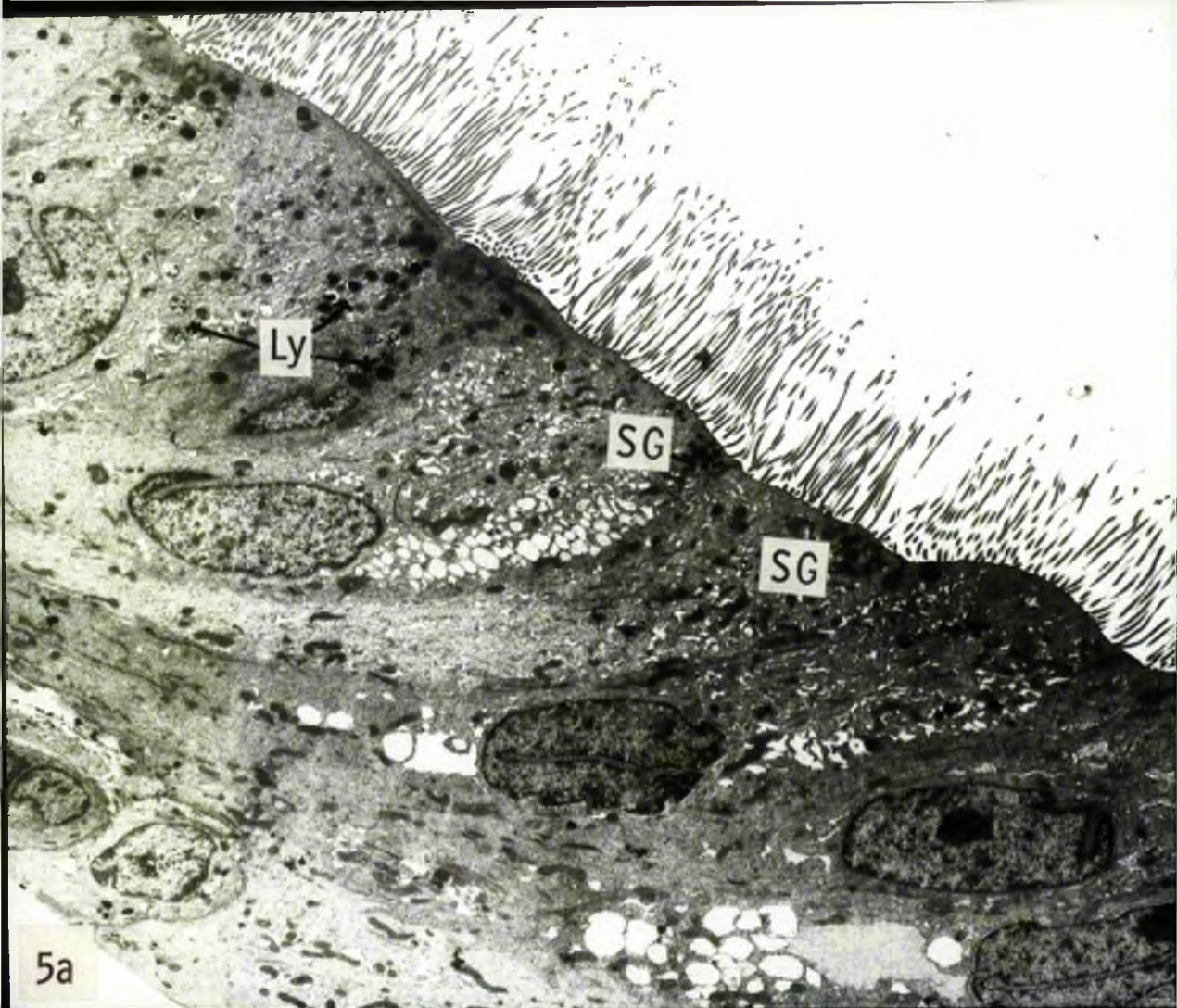


Figure 6: Lysosomes in rat oviductal epithelial cells.

a. Secretory cell. Gomori's Lead method for Acid  
Phosphatase. X23,670

Ly- lysosomes

SG- secretory granules

b. Ciliated cell. Gomori's Lead method for Acid  
Phosphatase. X23,670

Ly- lysosomes

N- nucleus

c. Ciliated cells. UA&LC. X23,670

GAL- giant autophagic lysosome

Ly- lysosomes



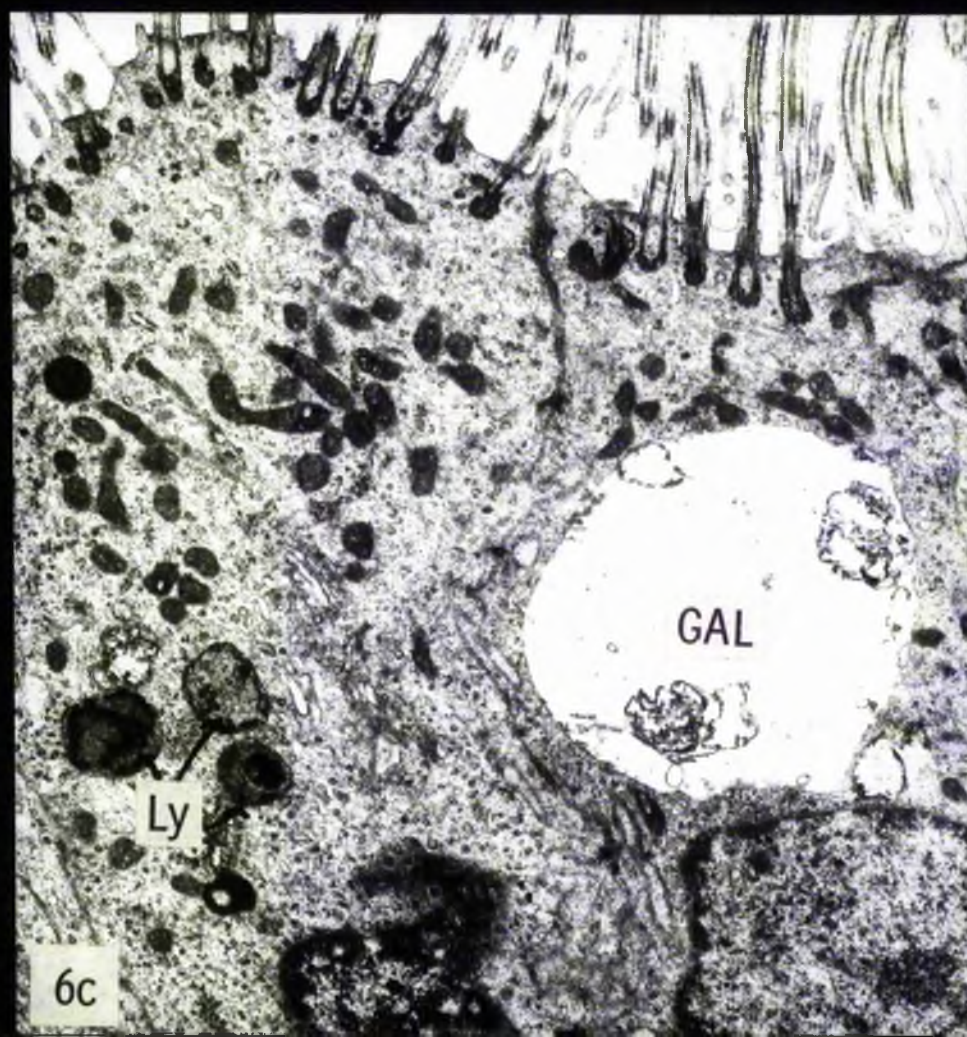
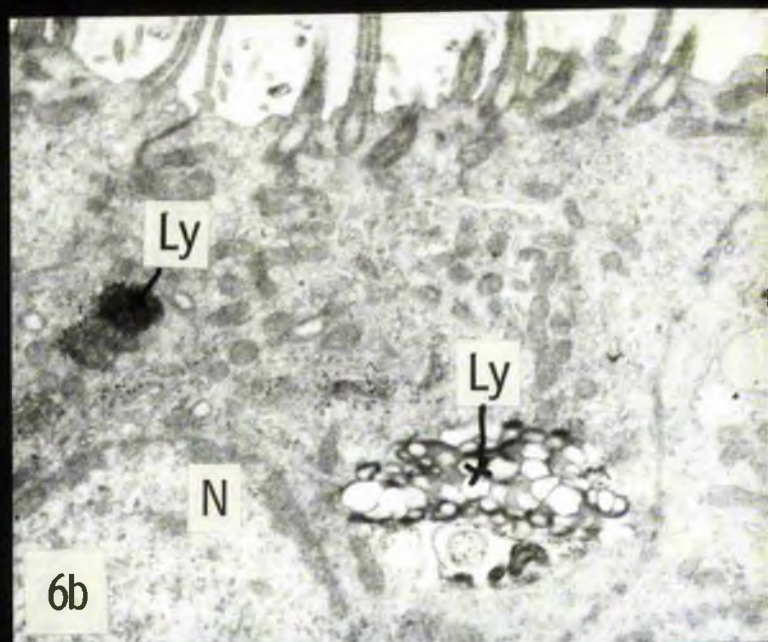
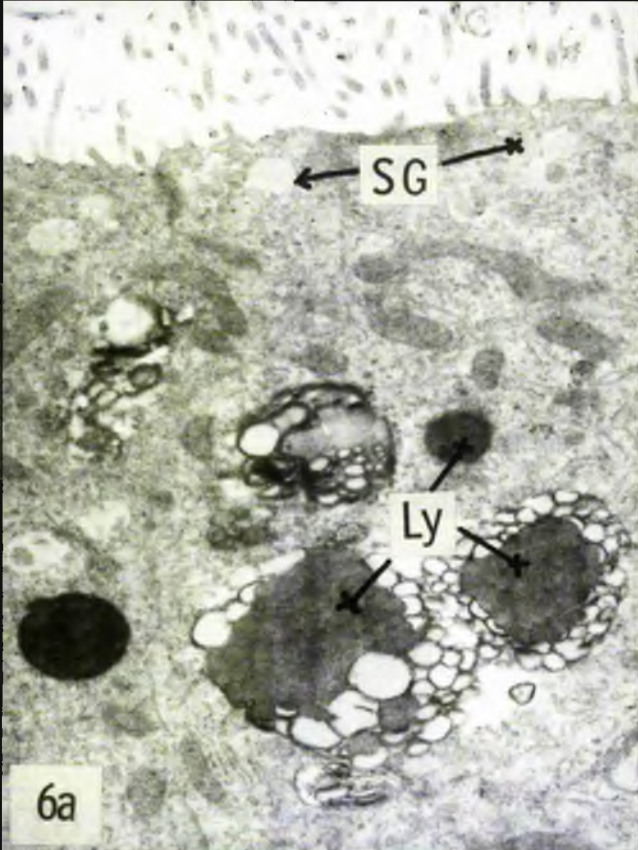


Figure 7: Rat preampulla. UA&LC. X3710

formation at arrow.

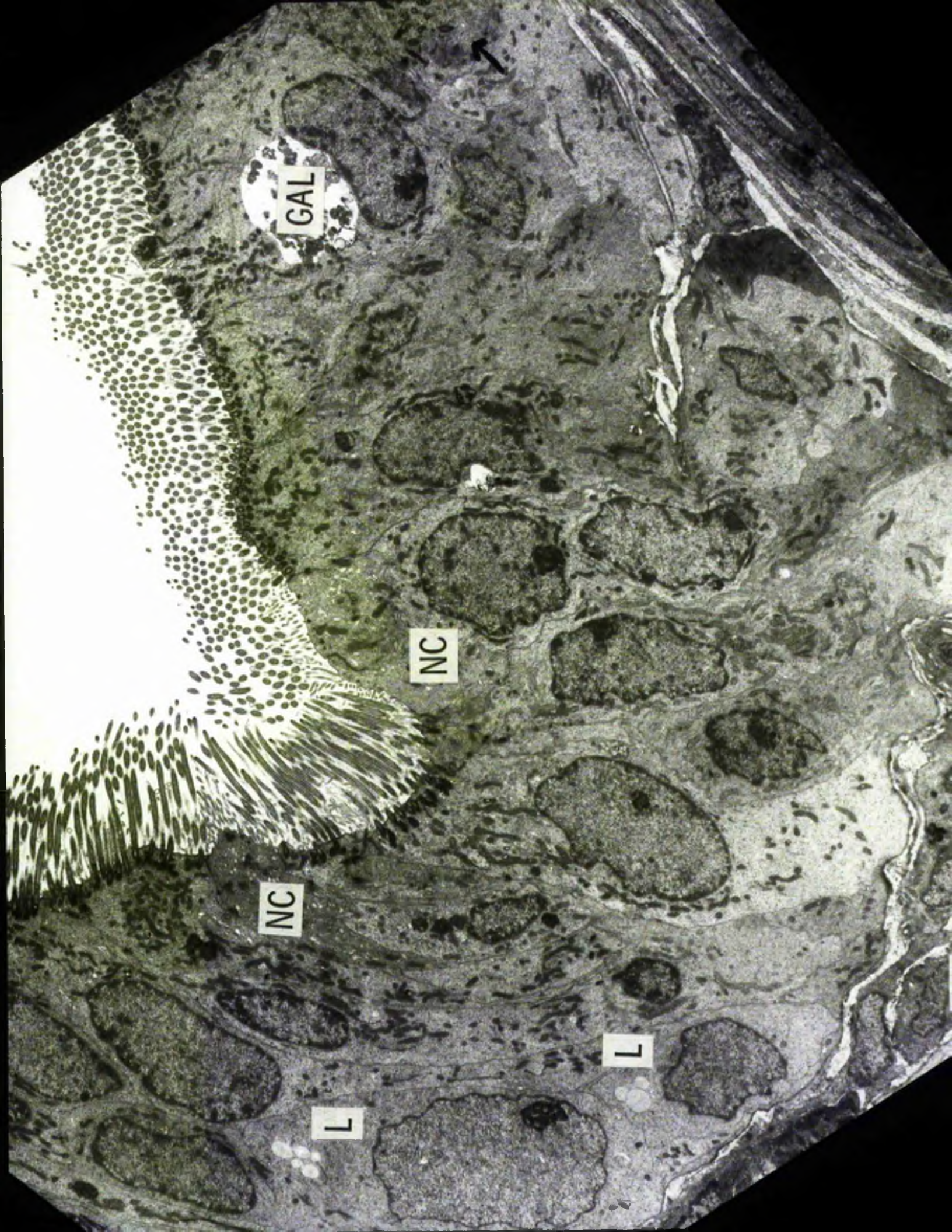
GAL- giant autophagic lysosome

L- lipid droplets

NC- nonciliated cell

Note: Early stage in ciliary vacuole





GAL

NC

NC

L

L



Figure 8: Rabbit ampulla containing non-granulated cells and  
ciliated cells. UA&LC. X3710

NC- nonciliated cell

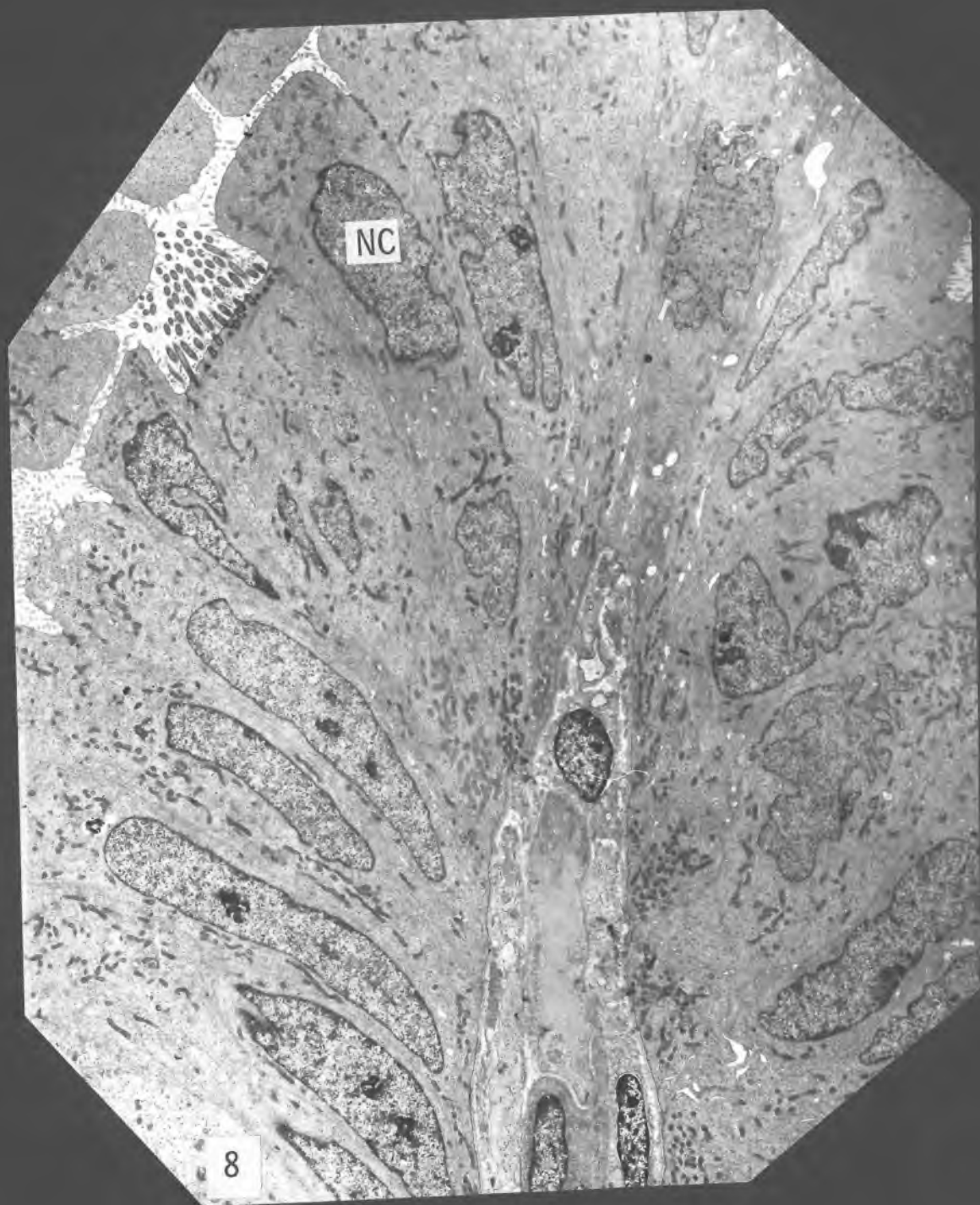


Figure 9: Secretion formation in the rabbit oviduct.

a. Formation of secretory granules. UA&LC. X40,000

G- Golgi apparatus

rer- rough endoplasmic reticulum

SG- secretory granule

b. Bases of secretory cells in rabbit isthmus.

UA&LC. X3430

BL- basal lamina

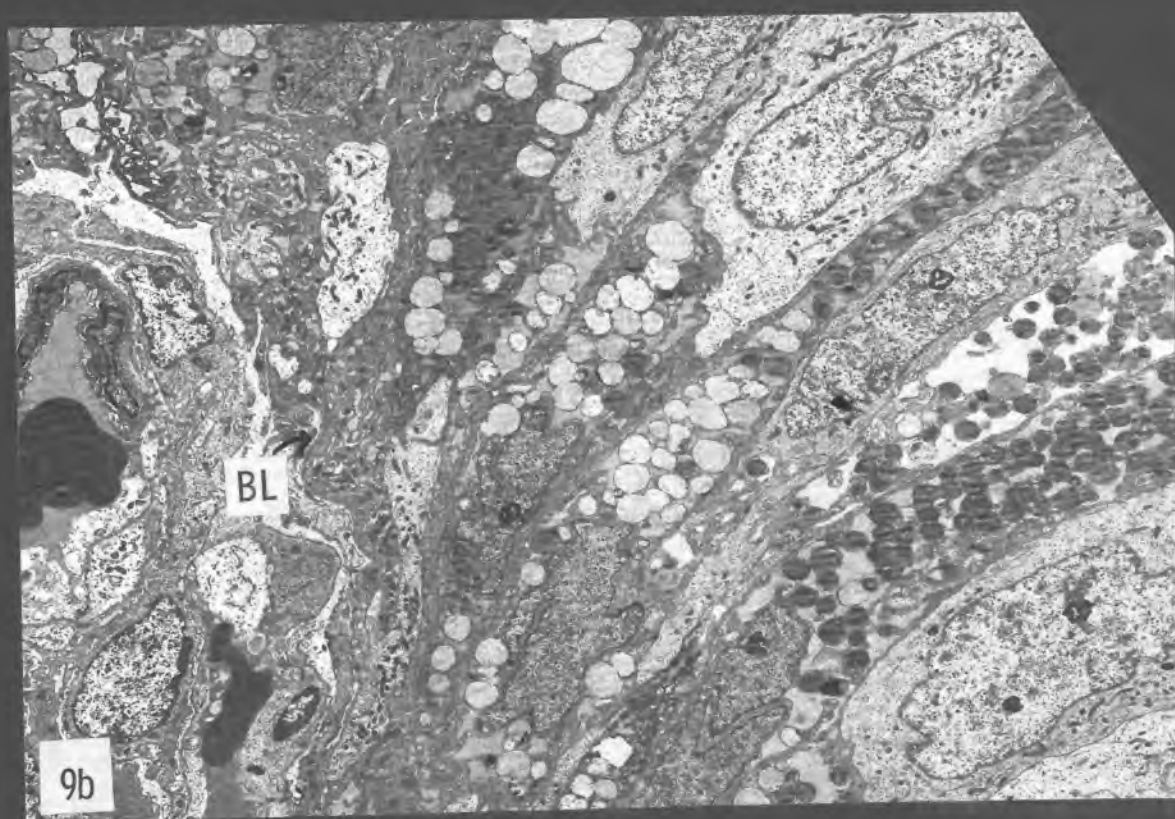
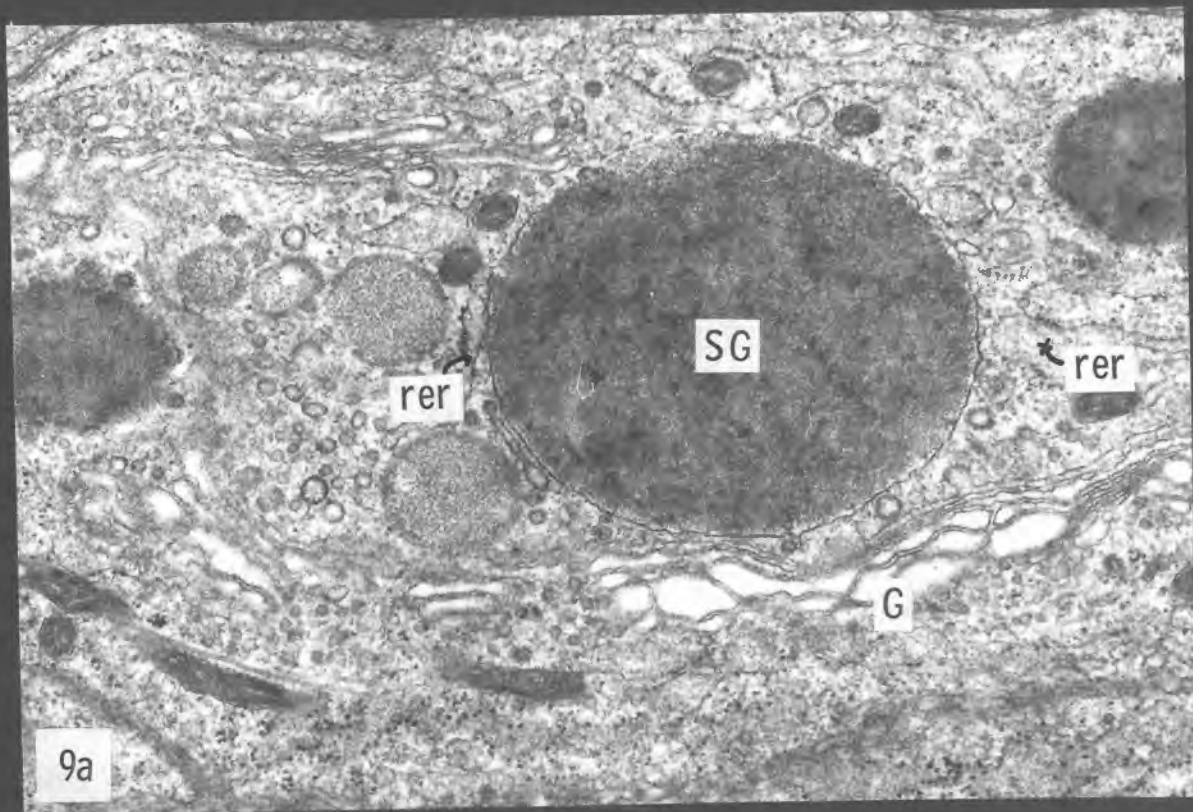


Figure 10: Asynchronous secretory cells in the rabbit oviduct.

a&b. UA&LC. X23,670



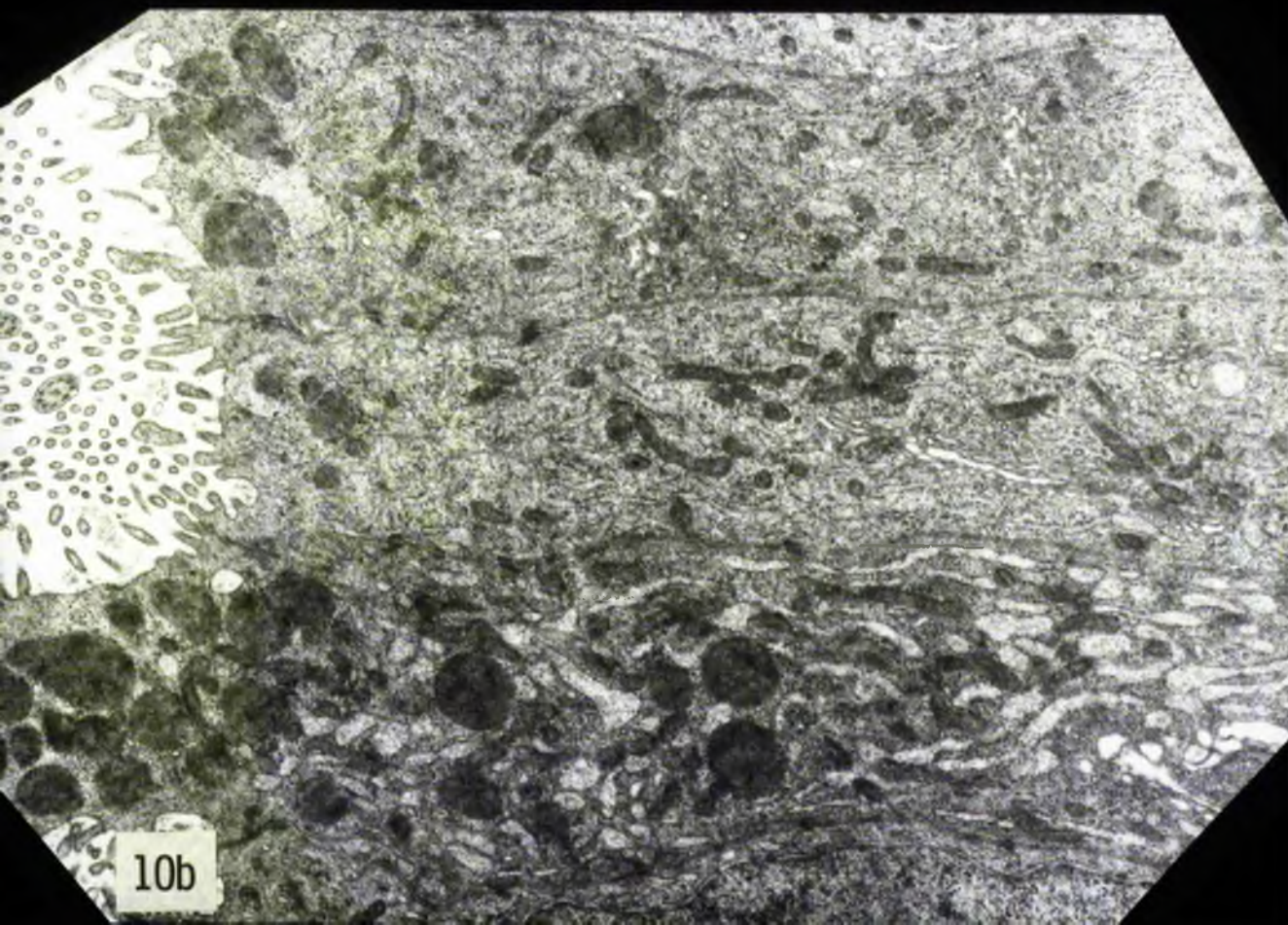
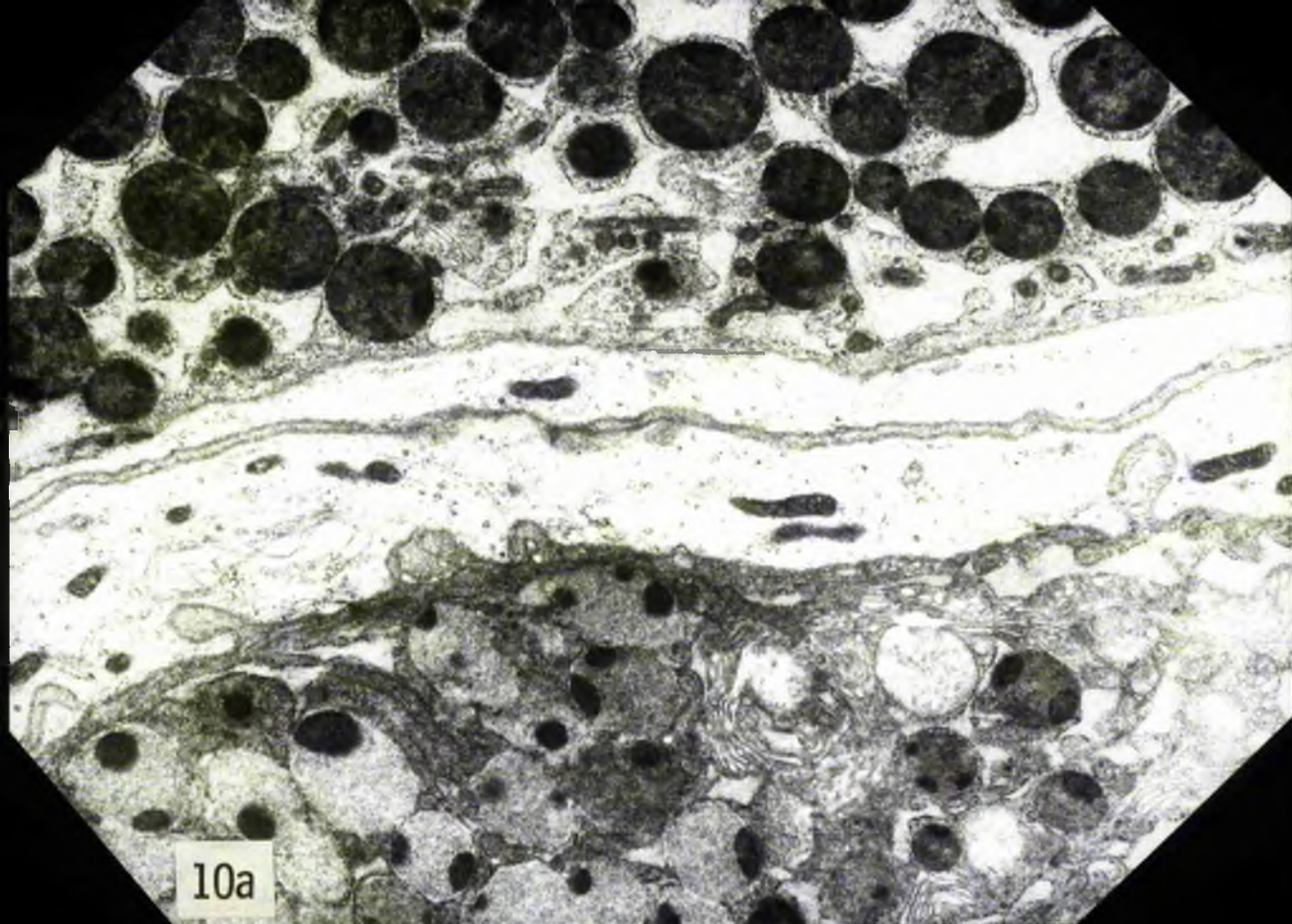


Figure 11: Rabbit ampulla.

a. At eight weeks of age. UA&LC. X3710



b. At eighteen months of age. UA&LC. X3710

GB- glycogen body



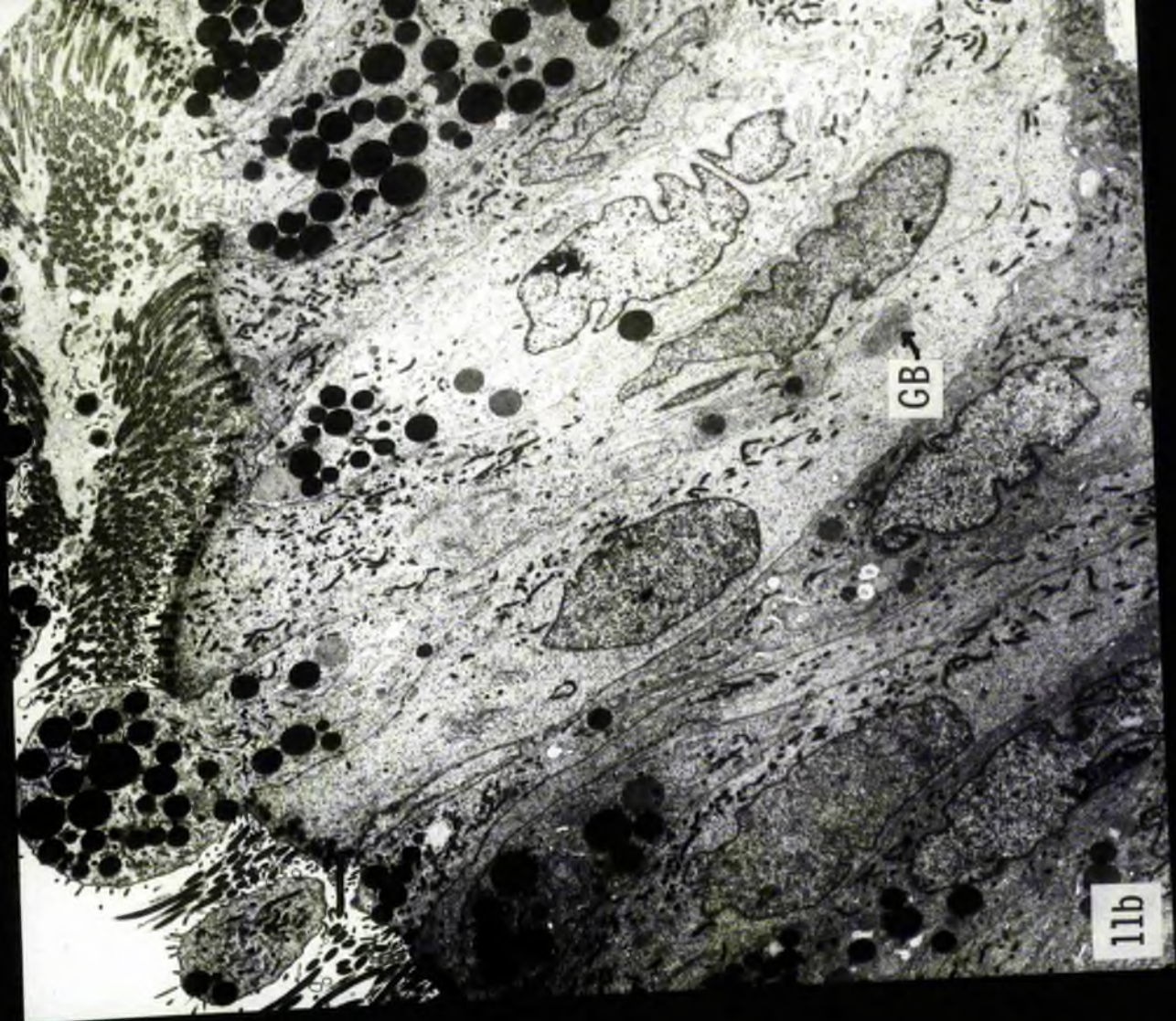




Figure 12: Oviductal cilia.

- a. Rabbit ciliated cells. UA&LC. X30,000  
k- knob-like appendages of basal bodies  
JC- junctional complex

- b. Rat ciliated cell. UA&LC. X40,000  
Note: The unusual occurrence of two basal feet  
on a single basal body.  
BF- basal feet

- c. Rat ciliated cell with two basal bodies exhibiting  
unusual bulbous rootlets (r). UA&LC. X40,000

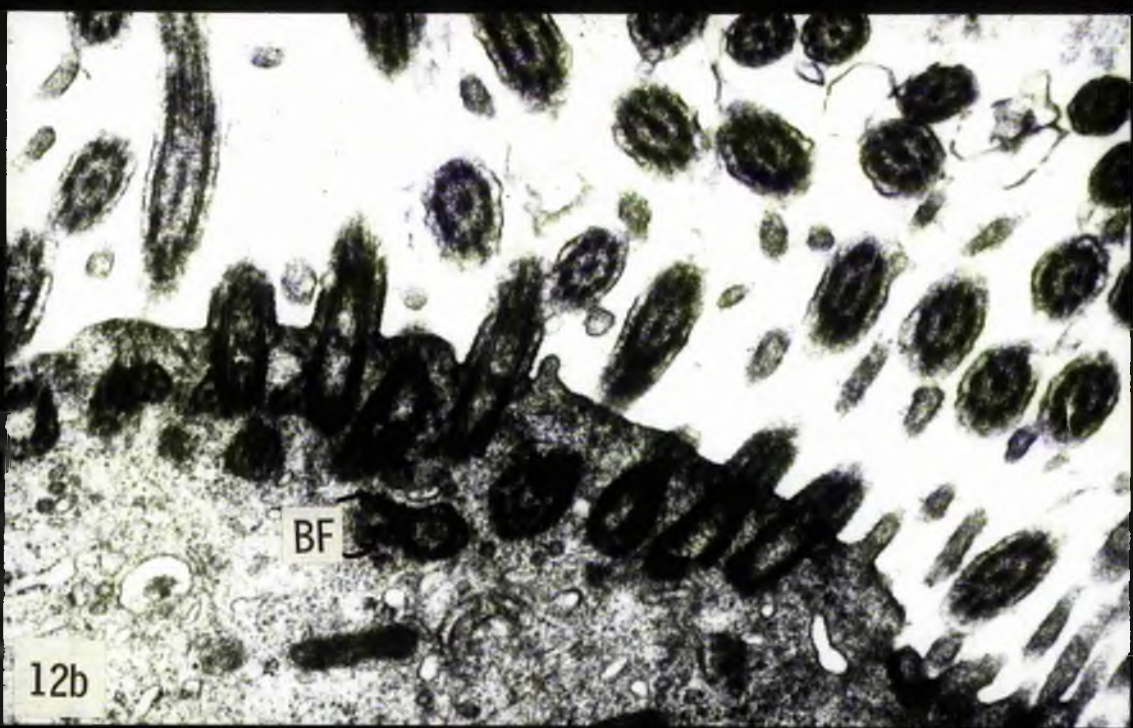
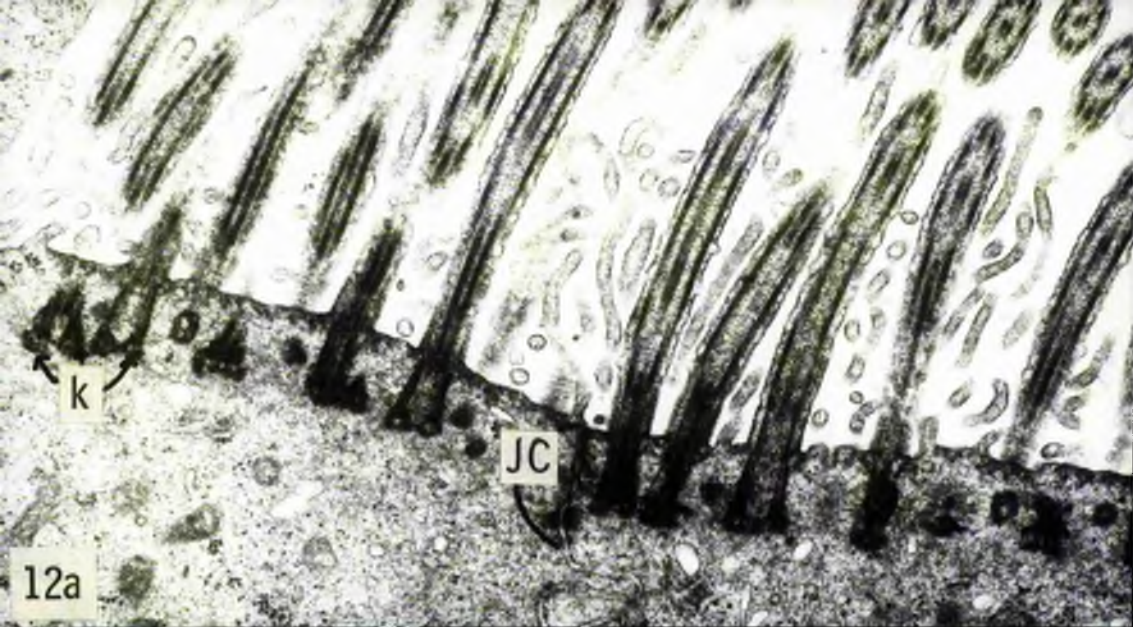


Figure 13: Glycogen bodies in rabbit oviductal ciliated cells.

a. Eight-week-old rabbit. UA&LC. X23,670

GB- glycogen body

b. Mature rabbit. UA&LC. X40,000



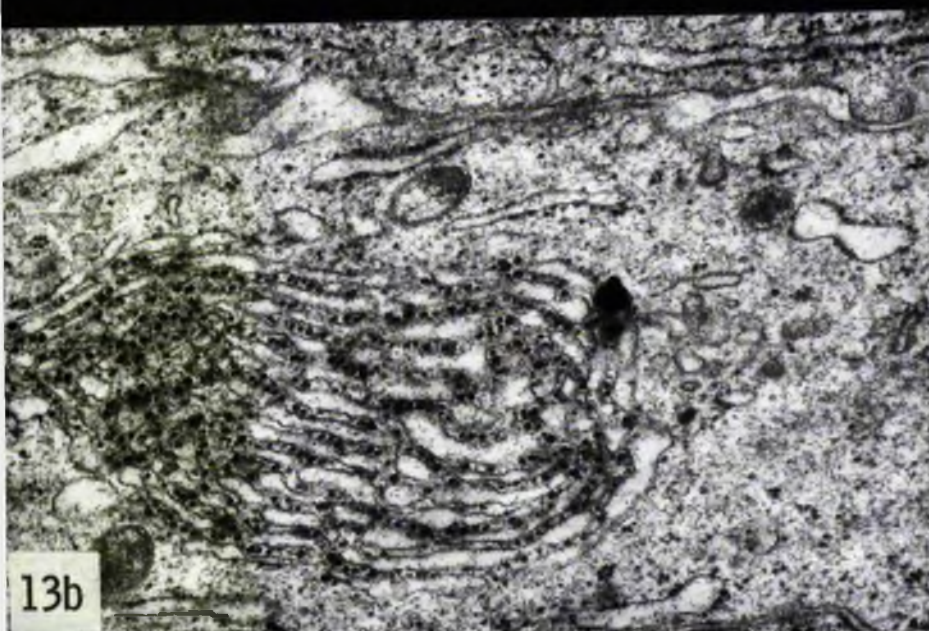
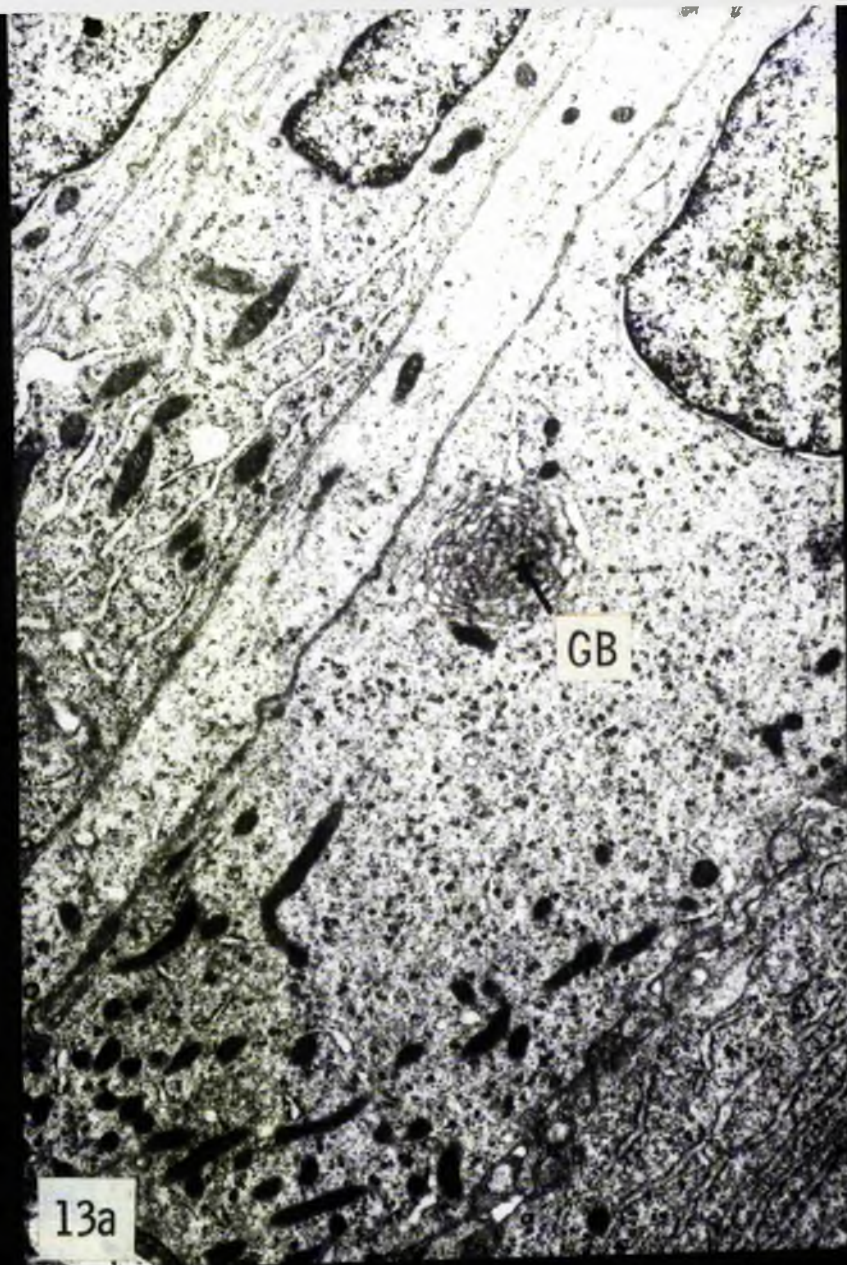


Figure 14: Mitochondrial inclusions in rat oviductal ciliated cells.

a. Cell containing mitochondria with inclusions and also uninvolved mitochondria (at arrows). UA&LC. X35,000

b. Light micrograph of ciliated cells containing mitochondria with inclusions (at arrows). Toluidine Blue. X1600

c. Longitudinal section of mitochondrial inclusion showing individual filaments. UA&LC. X100,000

g- In this region the globular subunits of the individual filaments are particularly clear

→ - mitochondrial cristae



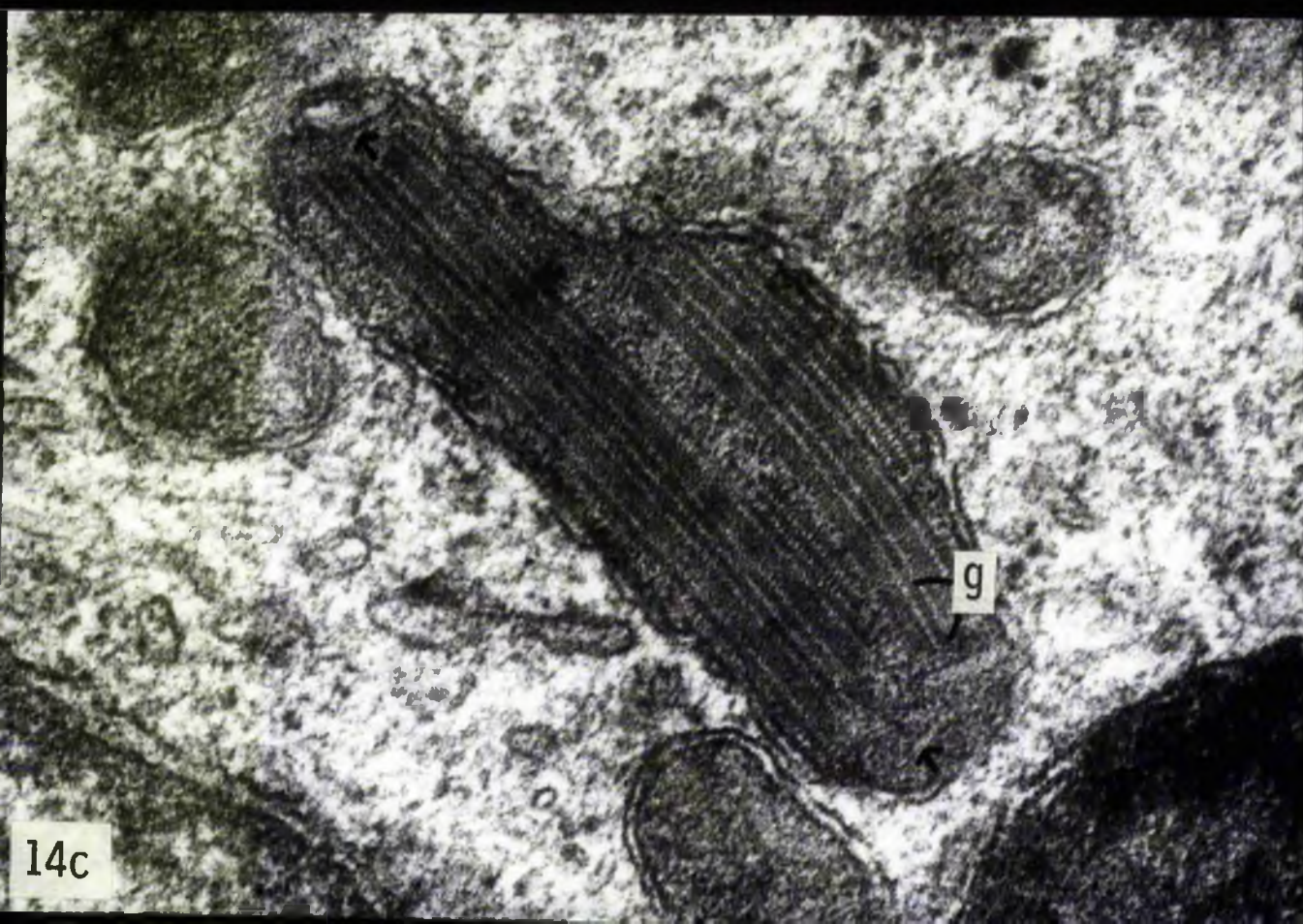
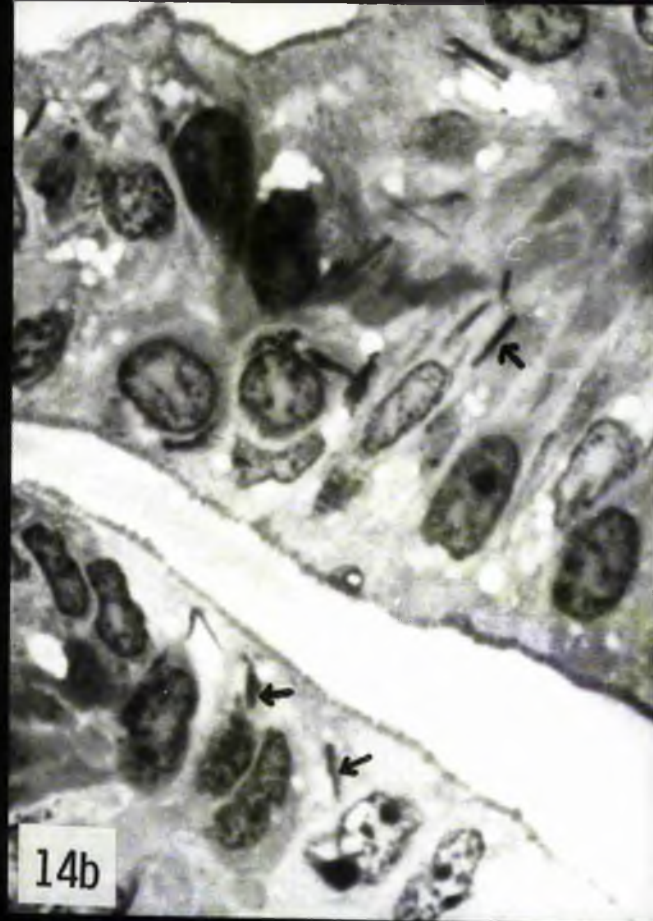
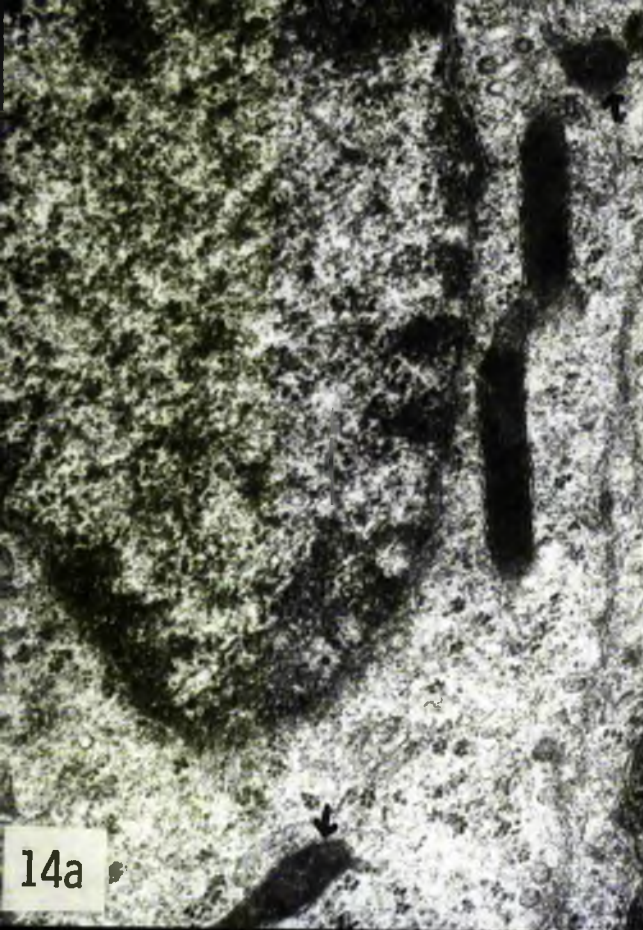




Figure 14 (continued):

- d. Cross section of mitochondrial inclusion, revealing hexagonal packaging of filaments (circled area).

UA&LC. X100,000

- e. Two cells containing several mitochondria with inclusions. UA&LC. X40,000

Note: The outer membranes of the mitochondria containing inclusions in the upper cell are difficult to distinguish due to the plane of section.

- f. Distortion of mitochondrial shape by the inclusions.

UA&LC. X40,000

- g. Mitochondrion partially distorted by the filamentous inclusion. UA&LC. X40,000



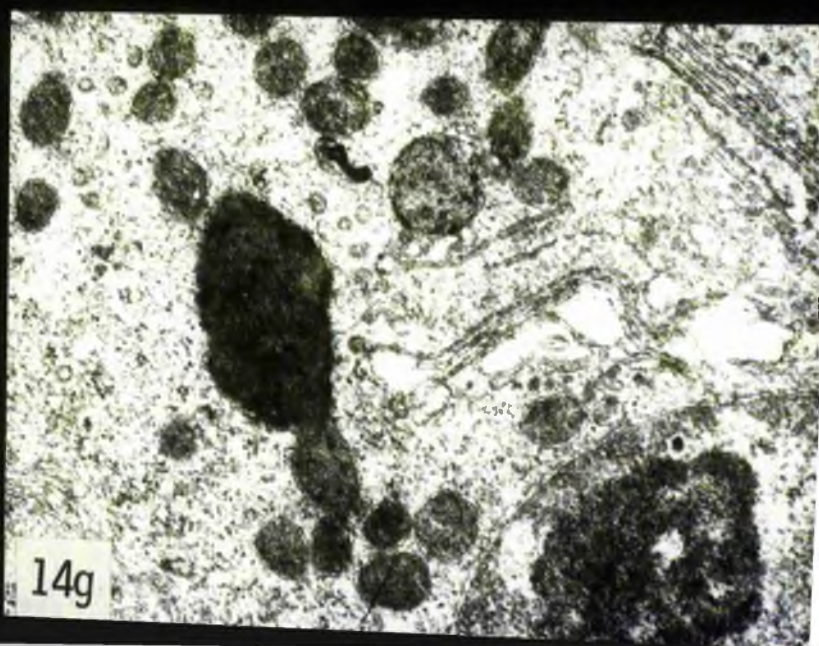
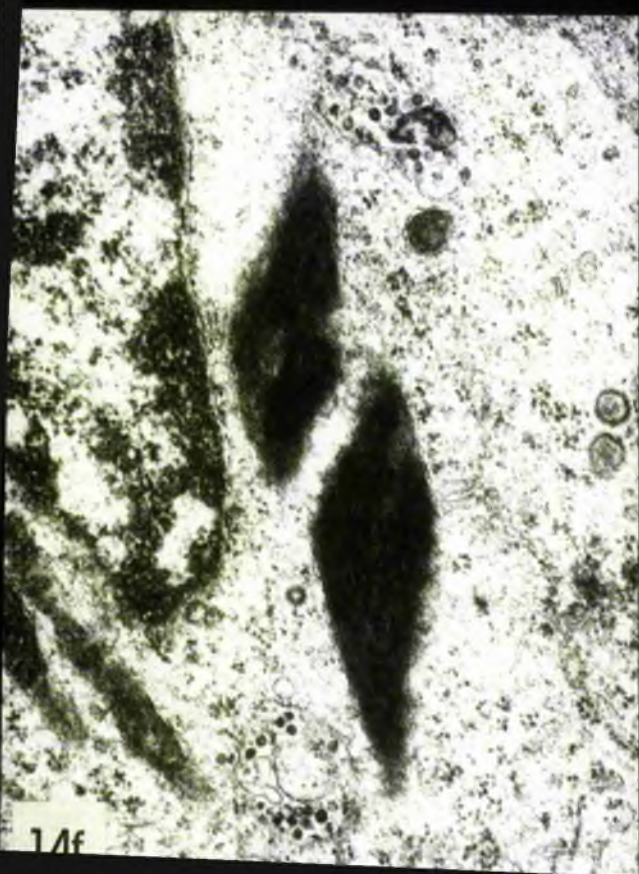
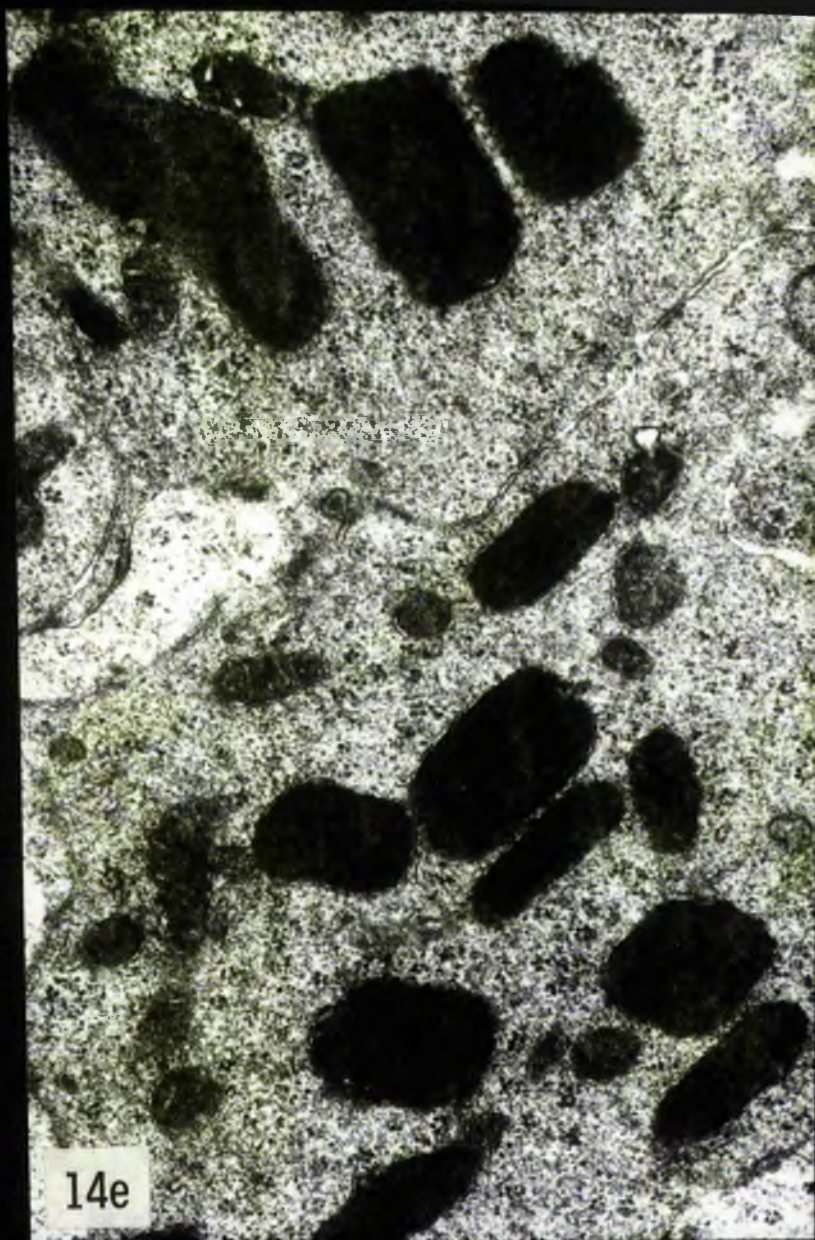




Figure 15: Ciliogenesis in the rat oviduct.

- a. Cell showing several stages in the process of ciliogenesis. UA&LC. X35,000

CF- condensation form

DC- developing centriole

PE- proliferative elements

- b. Cell in a later stage of ciliogenesis. Maturing centrioles have migrated to the apical membrane of the cell to become basal bodies and to form ciliary buds, basal feet and rootlets. UA&LC. X23,670

BB- basal body

BF- basal foot

CB- ciliary bud

CF- condensation form

PE- proliferative elements

r- rootlet

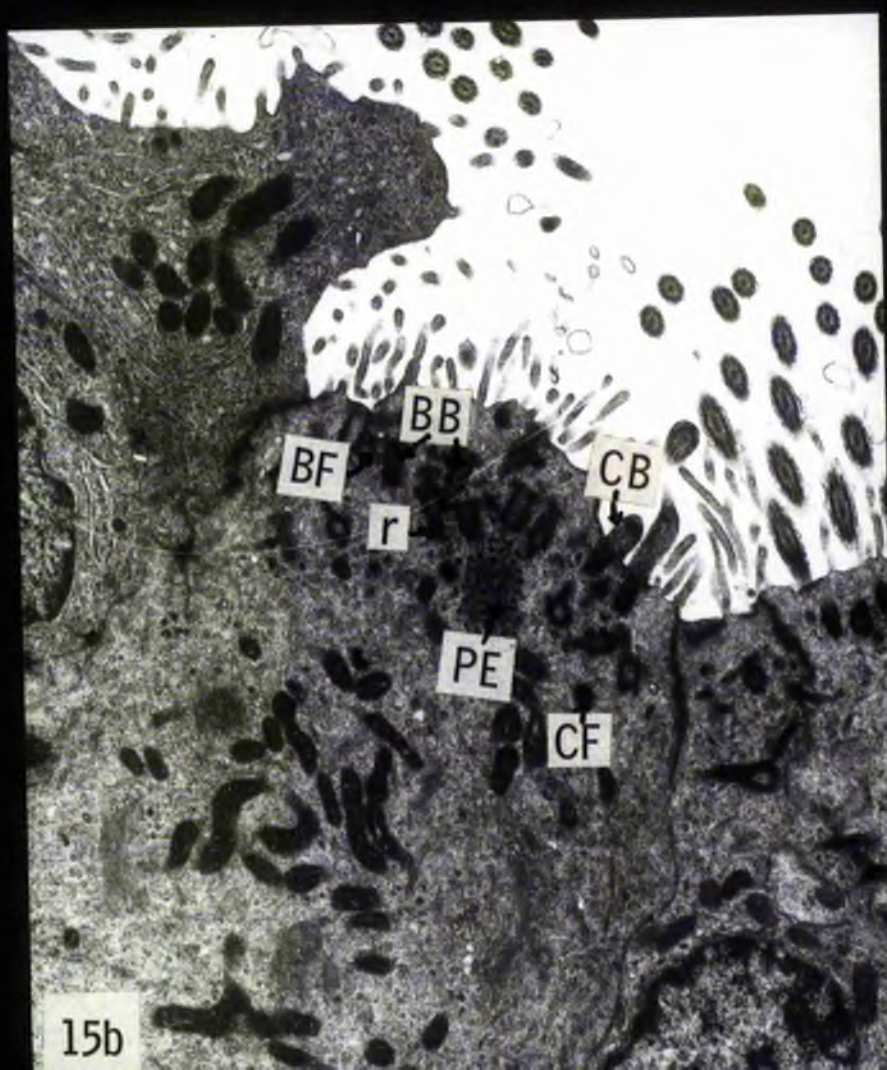
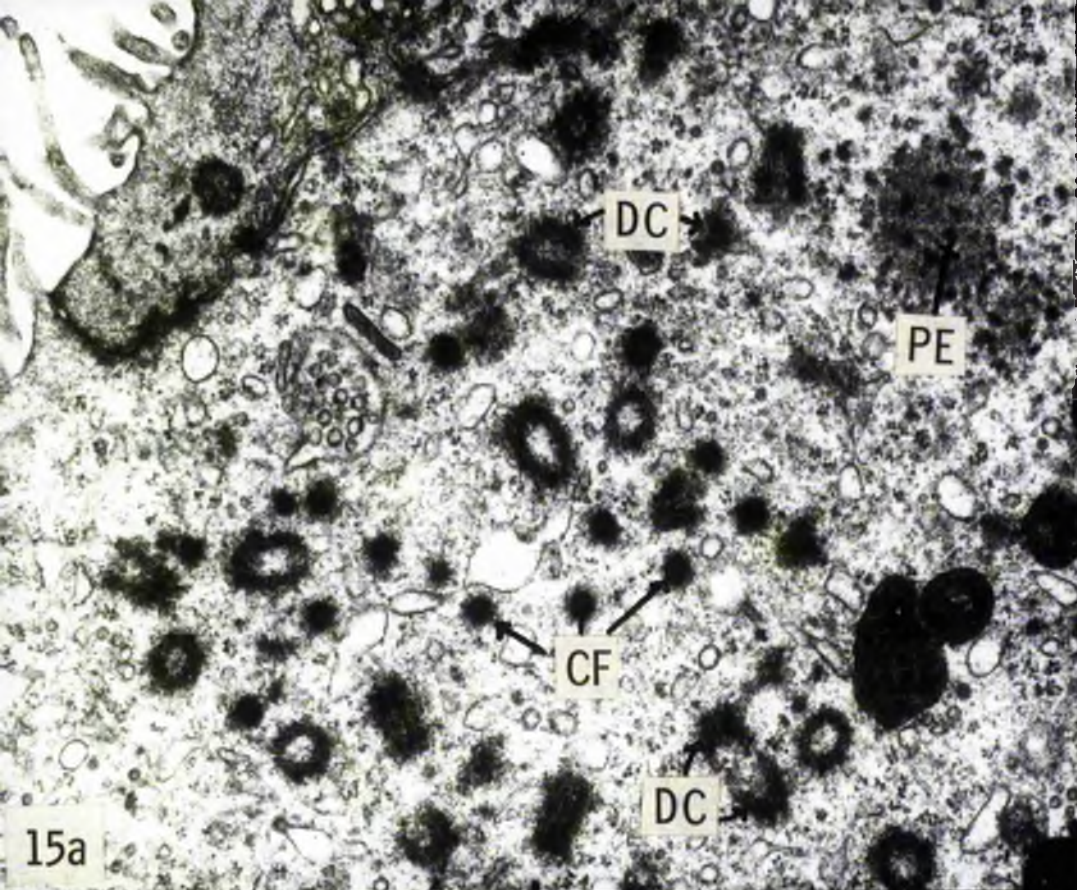


Figure 16: Ciliogenesis in the rabbit oviduct.

- a. Cell in the early stages of ciliogenesis showing short, regular microvilli and clusters of proliferative elements in the apical cytoplasm. UA&LC. X31,560

GC- generative complex

PE- proliferative elements

- b. More advanced stage of ciliogenesis showing formation of ciliary buds and the association of proliferative elements with the basal bodies. UA&LC. X23,670

CB- ciliary bud

CF- condensation form

PE- proliferative element



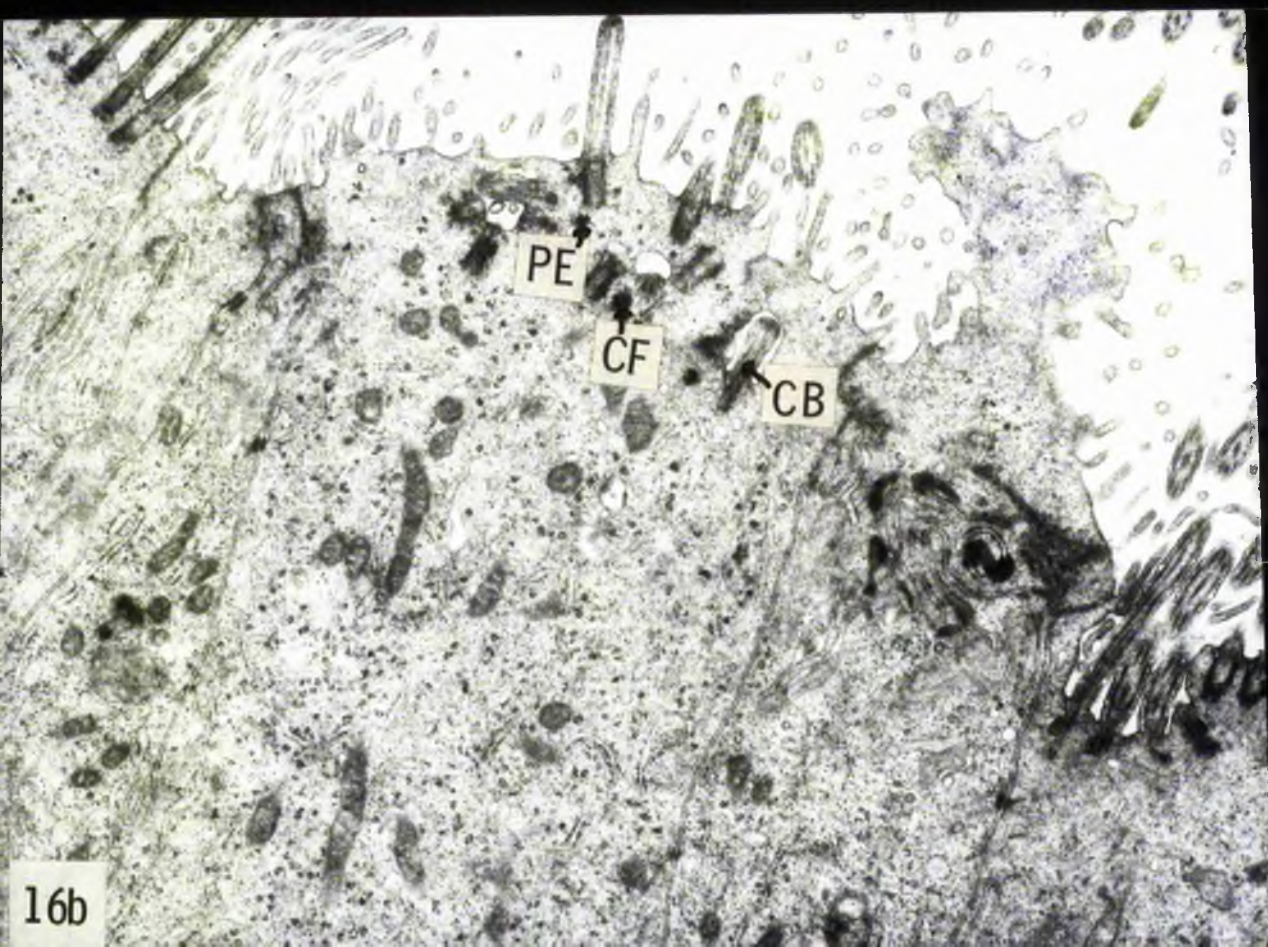
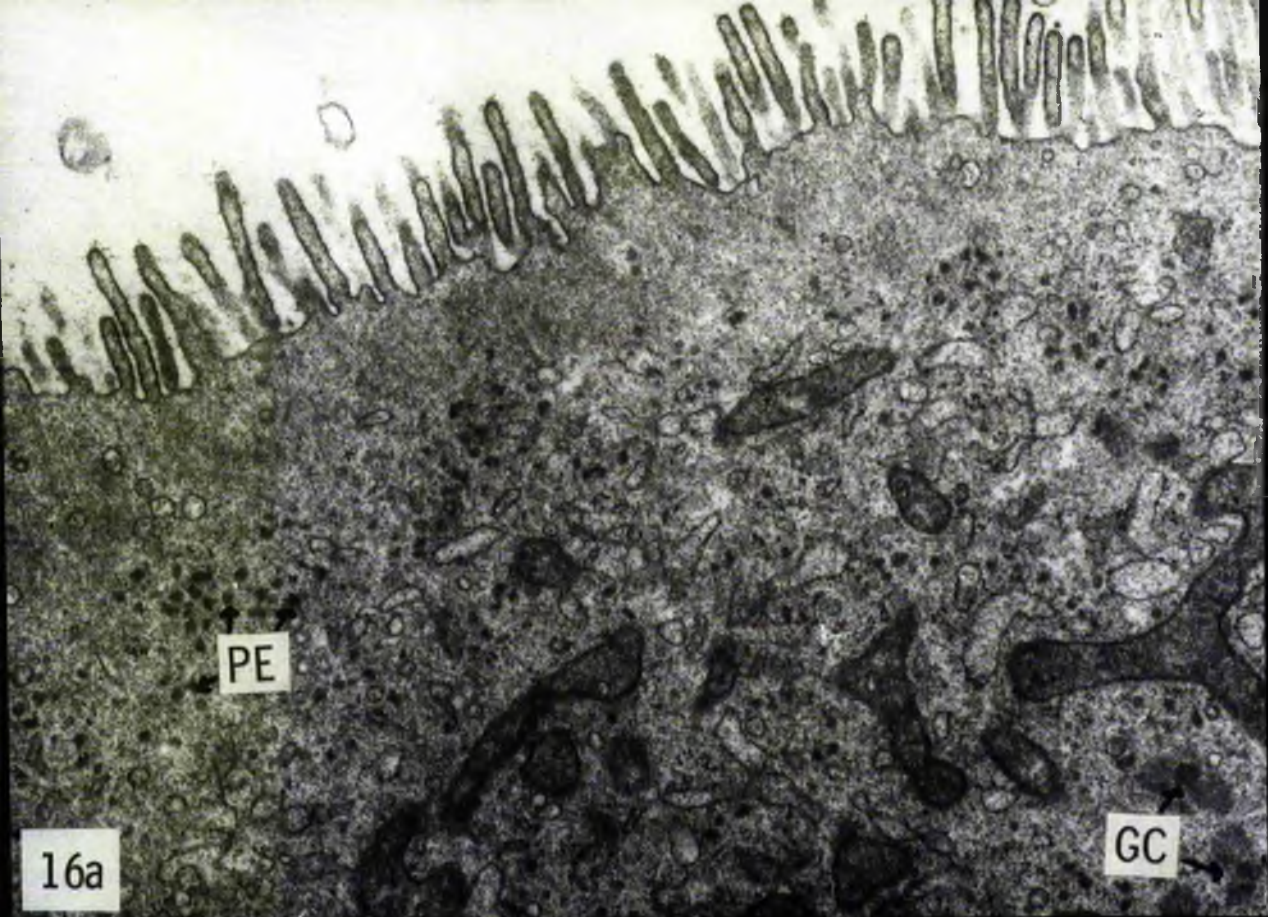


Figure 17: Ciliogenesis in the rabbit oviduct.

a. Unbuffered Silver Nitrate. X35,000

CC- ciliogenic cell  
r- free ribosomes  
PE- proliferative elements  
p- polyribosomes  
rer- rough endoplasmic reticulum  
SC- secretory cell

b. Enlargement of boxed area in 'a'. Unbuffered Silver Nitrate. X87,500

p- polyribosomes  
PE- proliferative elements  
BB- basal body



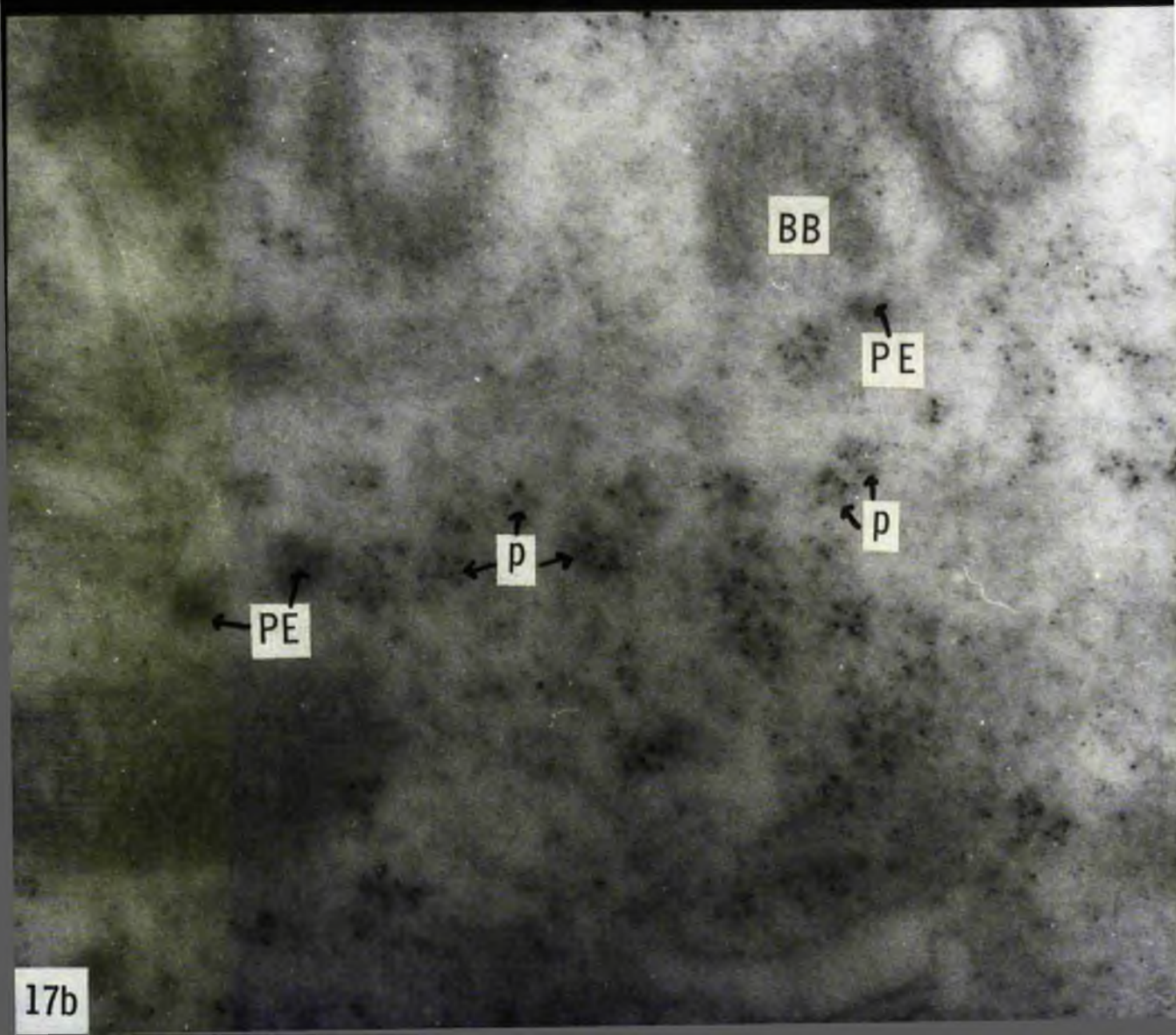
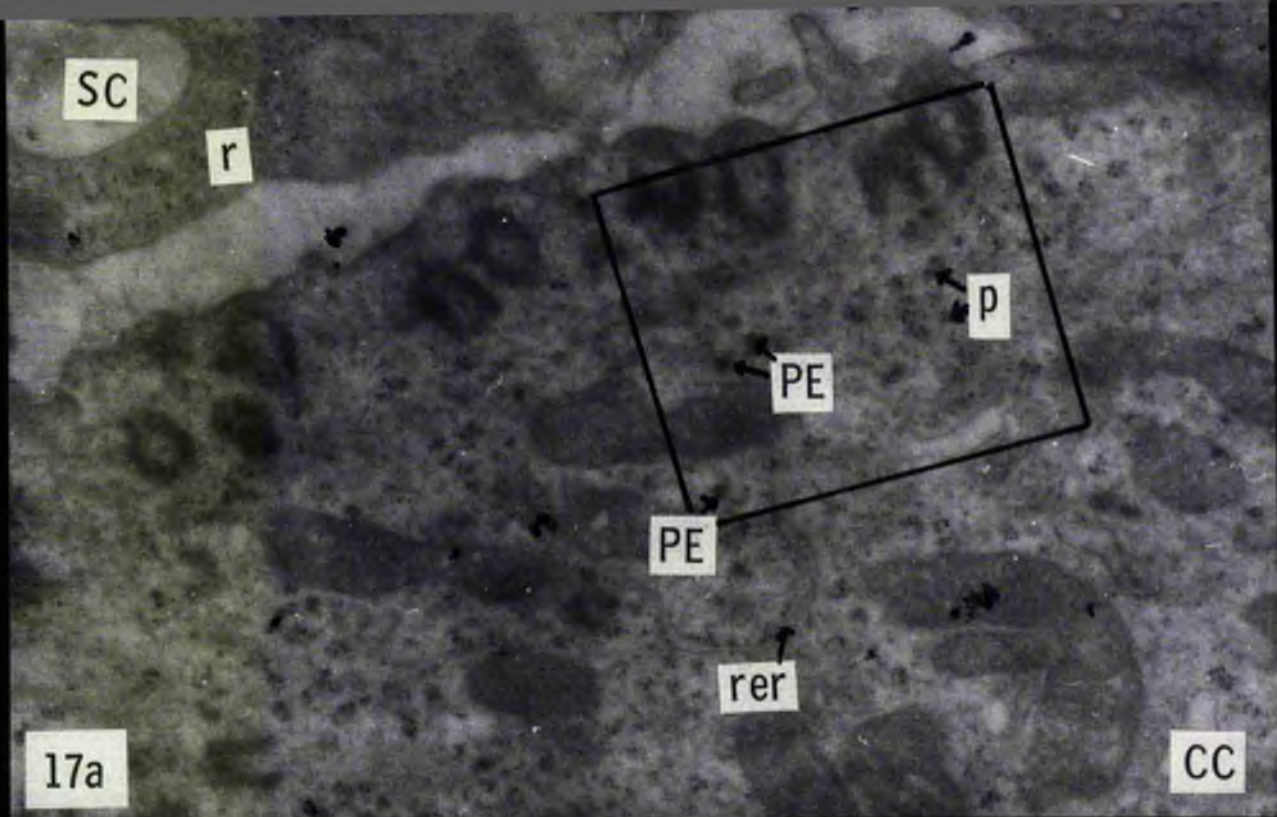




Figure 18: Ciliary vacuoles in 'basal' epithelial cells.

a. Eight-week-old rabbit oviduct. UA&LC. X5580

BL- basal lamina

CV- ciliary vacuole

rer- rough endoplasmic reticulum

→ - ciliary bud forming in distended segment of rer

b. Three-week-old rat oviduct. UA&LC. X27,615

BL- basal lamina

CV- ciliary vacuole

f- cytoplasmic fibrils

PV- primary ciliary vesicle

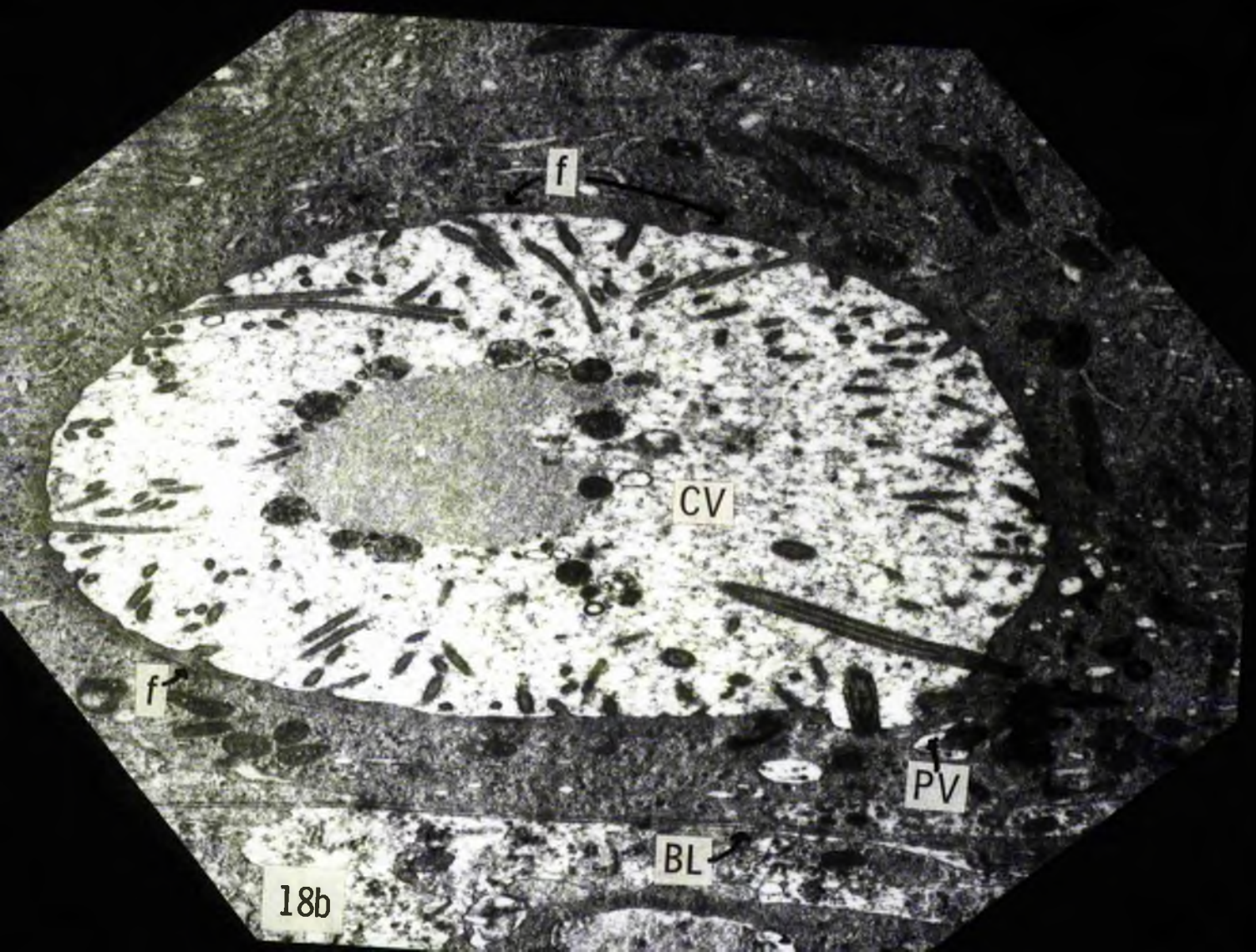
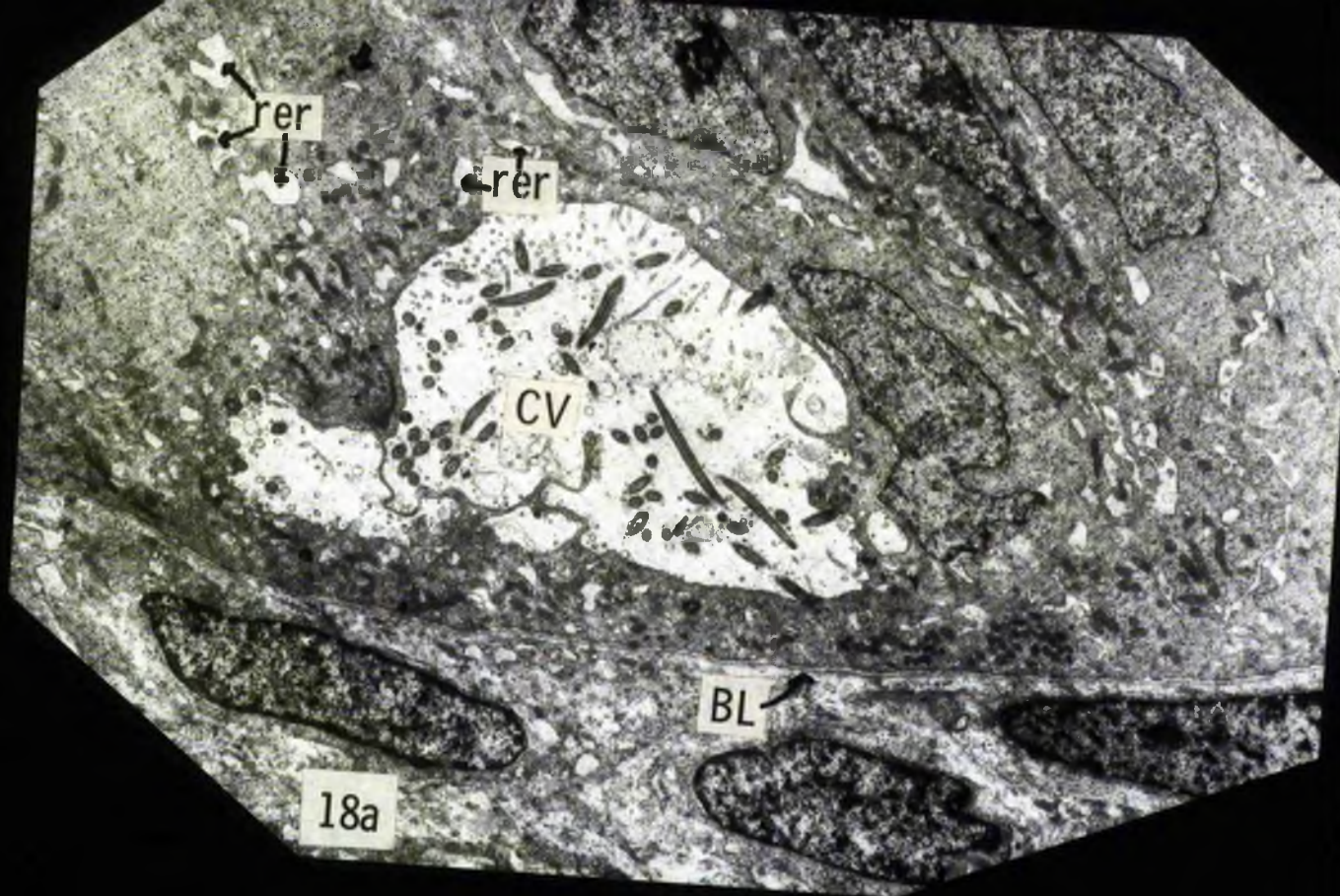




Figure 19: Ciliary vacuoles in the rat oviduct.

- a. Rat preampulla showing ciliary vacuoles in different stages in development. UA&LC. X3710

BL- basal lamina

CC- ciliogenic cell

CV1- ciliary vacuole in early stage of development showing clusters of basal bodies and primary ciliary vesicles

CV2- ciliary vacuole in later stage of development

- b. Enlargement of ciliary vacuole (CV2) showing that most of the primary ciliary vesicles have fused and that cilia have grown from the basal bodies into the interior of the vacuole. UA&LC. X23,670

BF- basal foot

PV- primary ciliary vesicle

r- rootlets

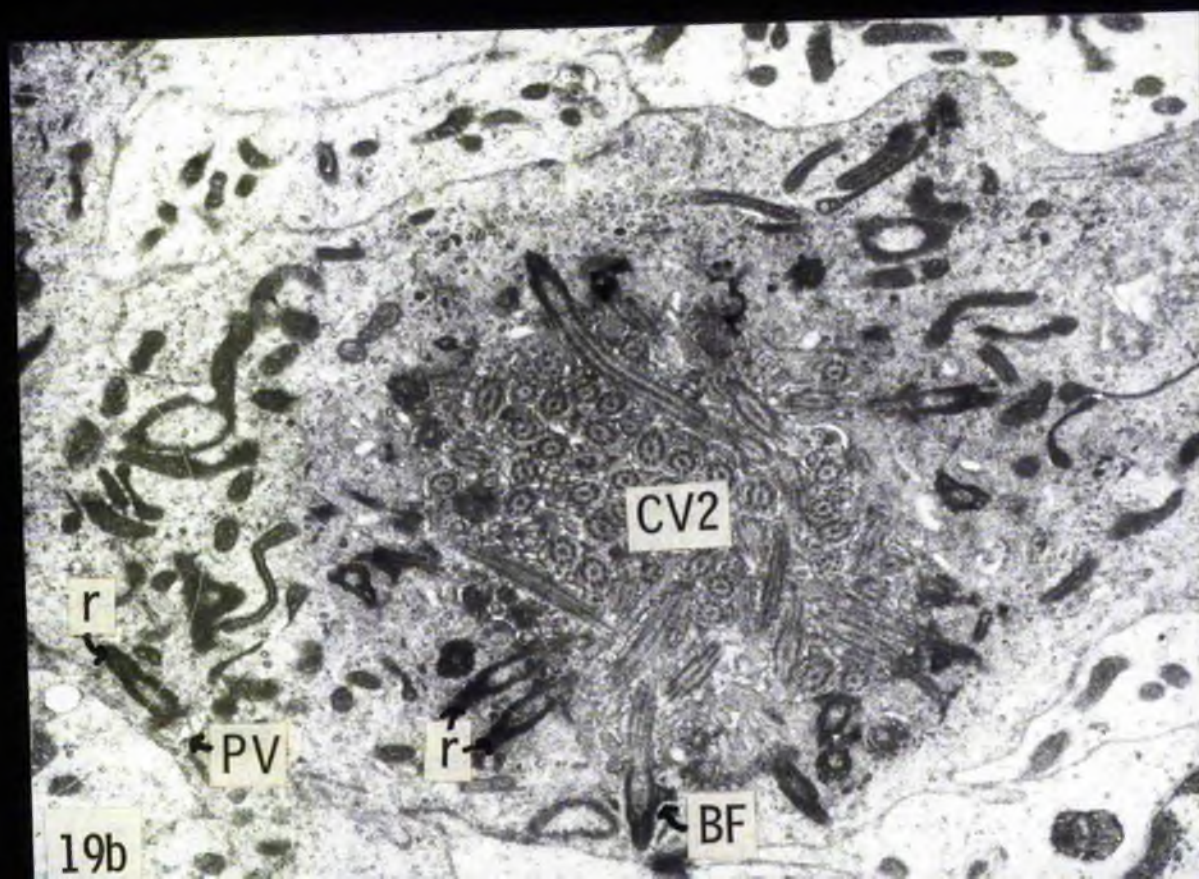
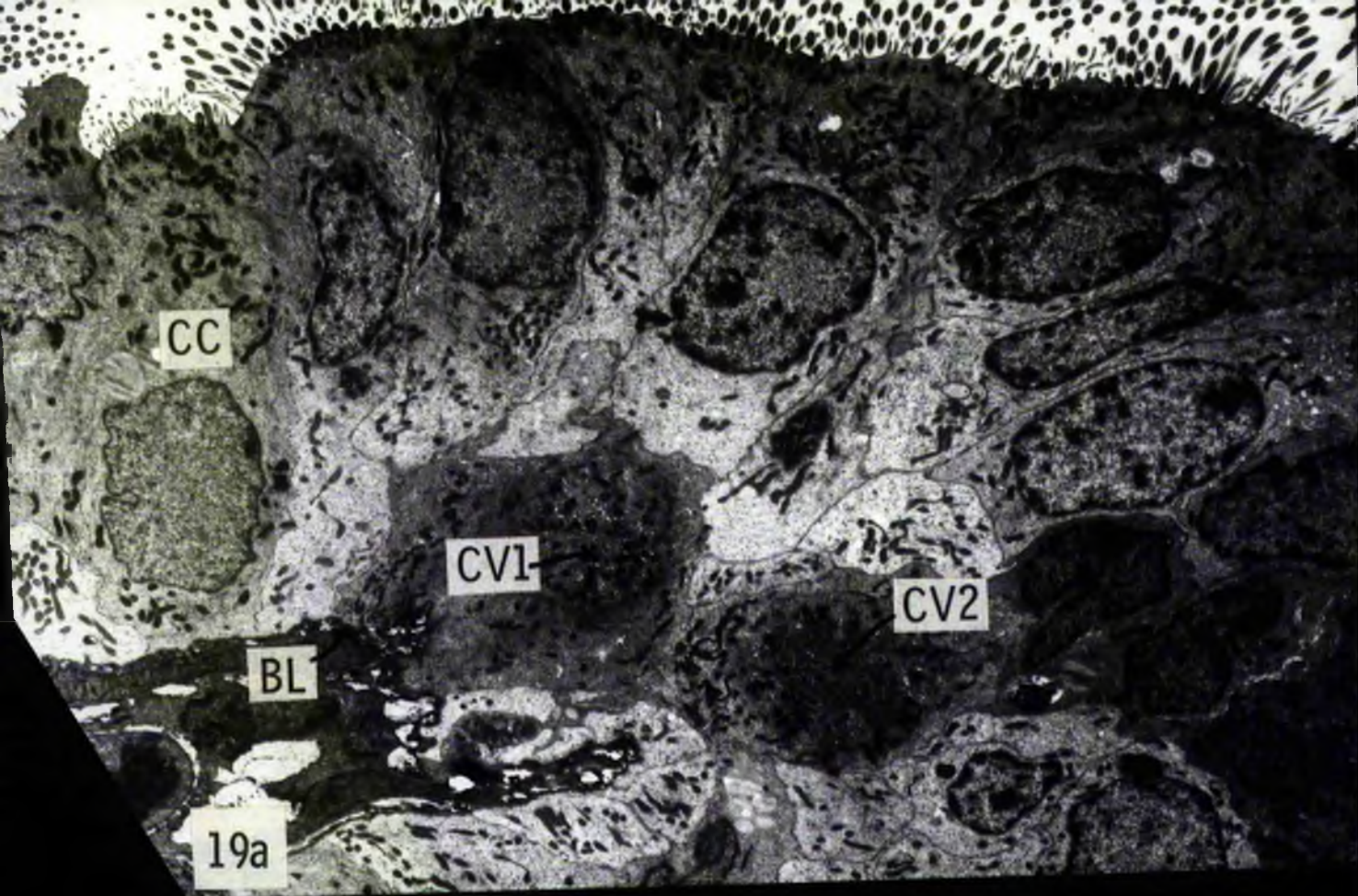


Figure 20: Ciliary vacuoles in the rat oviduct.

a. Ciliary vacuole in a 'basal' epithelial cell.

UA&LC. X6510

BL- basal lamina

CV- ciliary vacuole

f- cytoplasmic fibrils

m- cell membrane

b. Ciliary vacuole. Gomori's Lead method for Acid

Phosphatase. X23,670

CV- ciliary vacuole

Ly- lysosomes





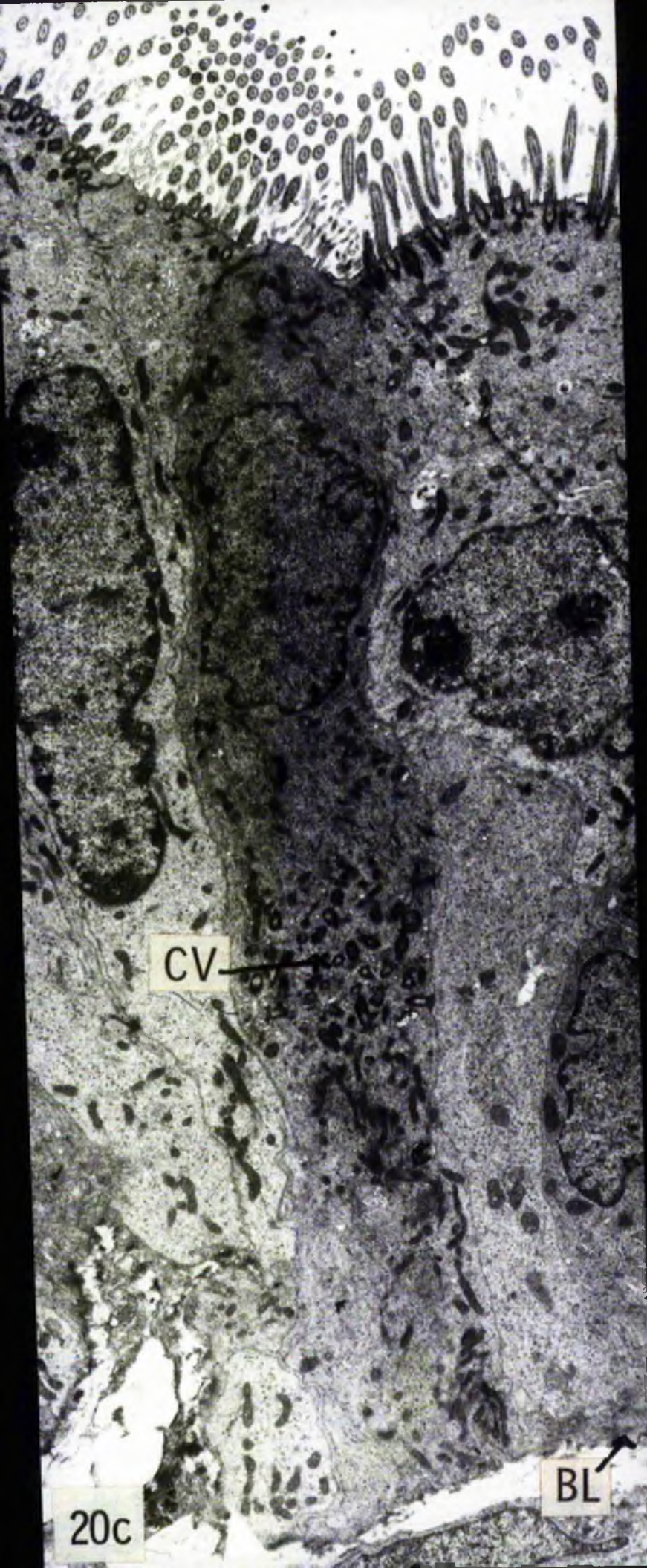
Figure 20 (continued):

c. Early stage in ciliary vacuole formation in  
a columnar nonciliated cell. UA&LC. X6510

BL- basal lamina

CV- ciliary vacuole





CV

BL

20c



Figure 21: Rat preampulla.

a. UA&LC. X3710

GAL- giant autophagic lysosome

LP- lamina propria

Lym- lymphocyte

NC- nonciliated cells

PE- proliferative elements

b. Enlargement of part of 'a' showing lymphocyte in  
intercellular space and proliferative elements in  
basal part of cell. UA&LC. X23,670

BL- basal lamina

Lym- lymphocyte

PE- proliferative elements

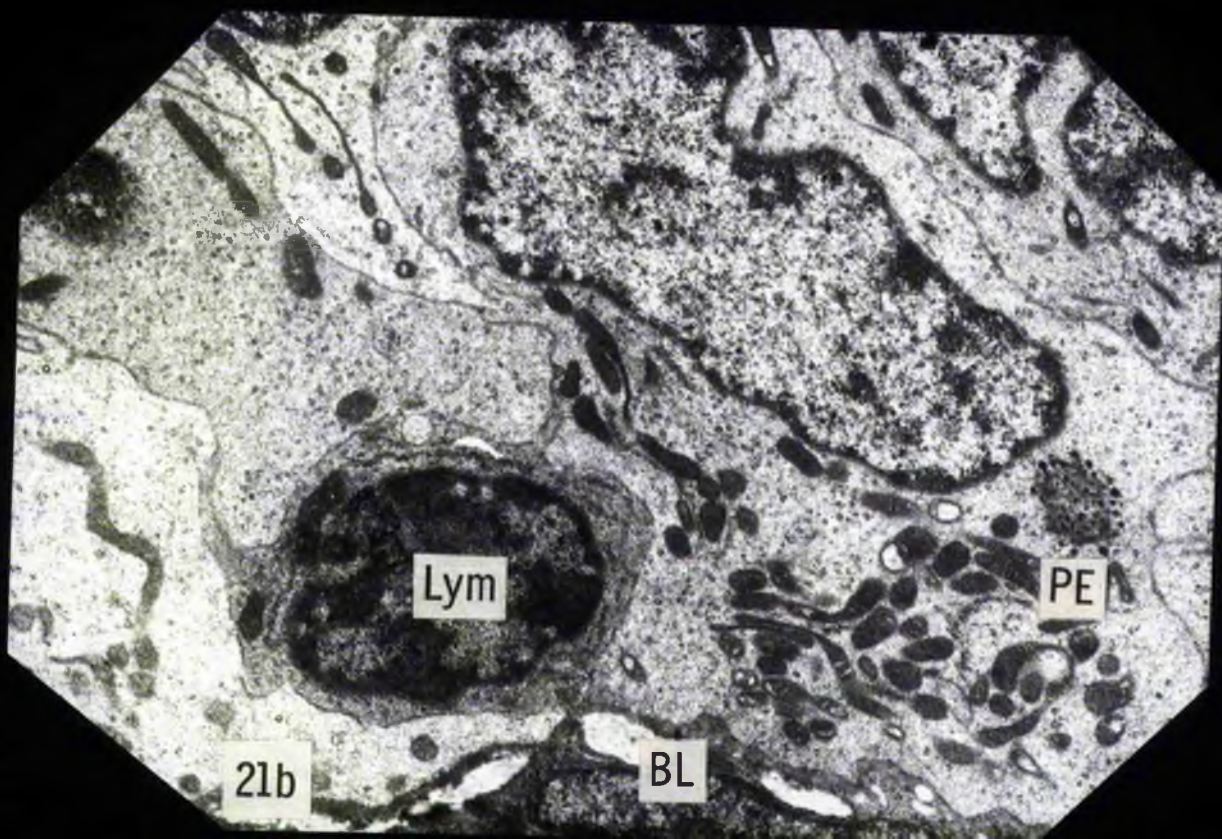
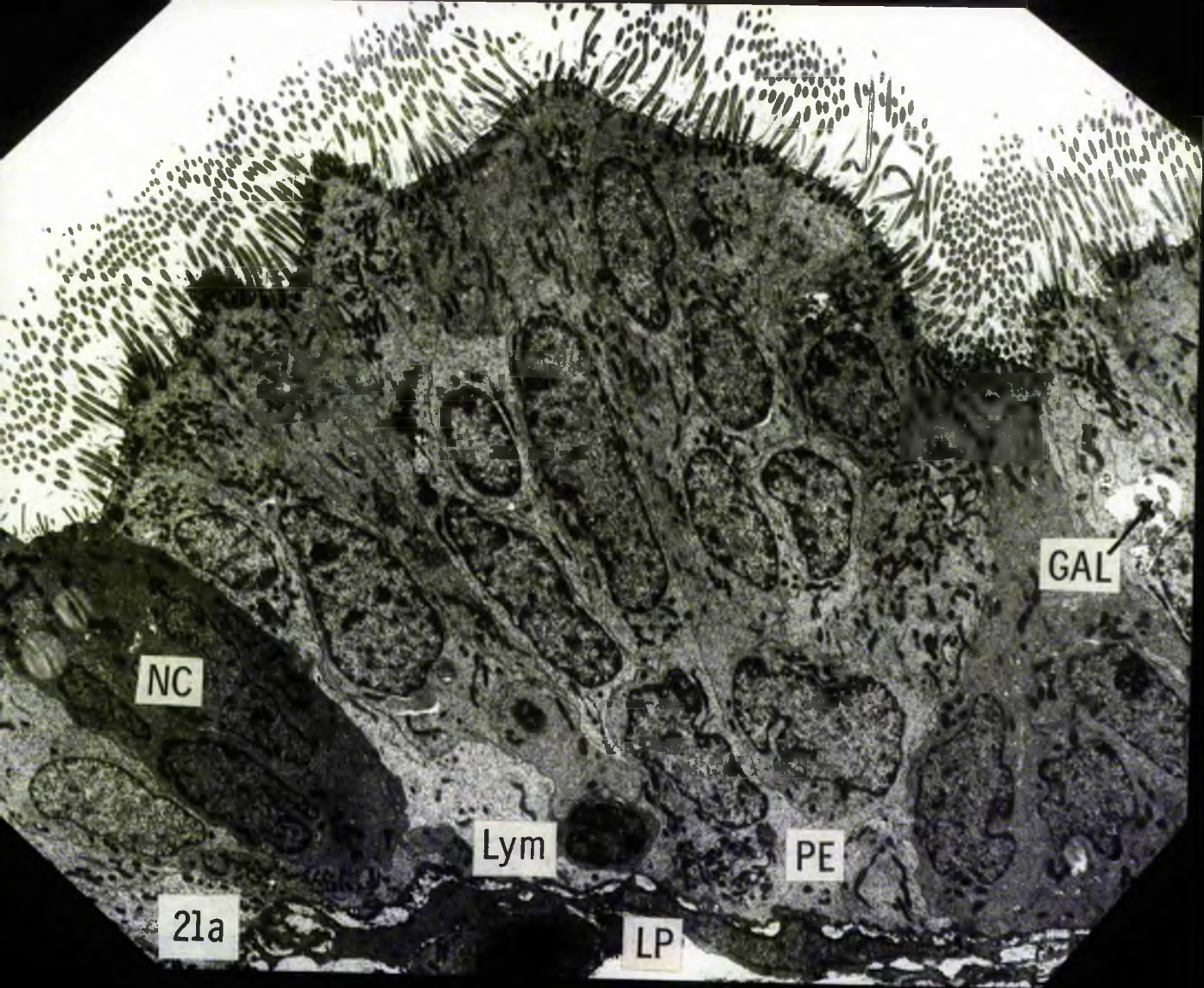


Figure 22: Rabbit ciliated cell containing a single secretory granule(SG). UA&LC. X40,000

Figure 23: Rabbit oviductal epithelium containing a degenerating ciliated cell (DC). Toluidine Blue. X2000



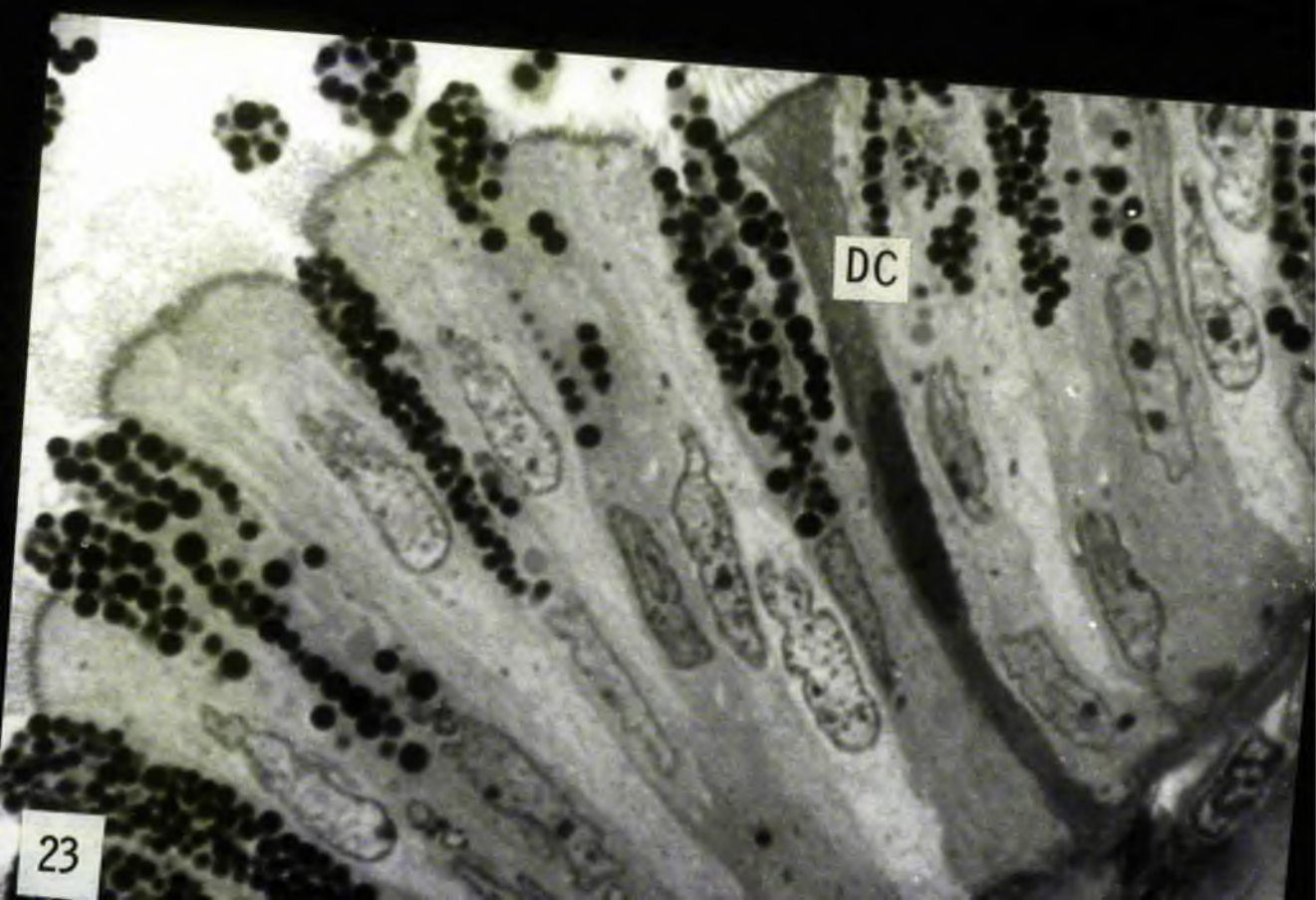
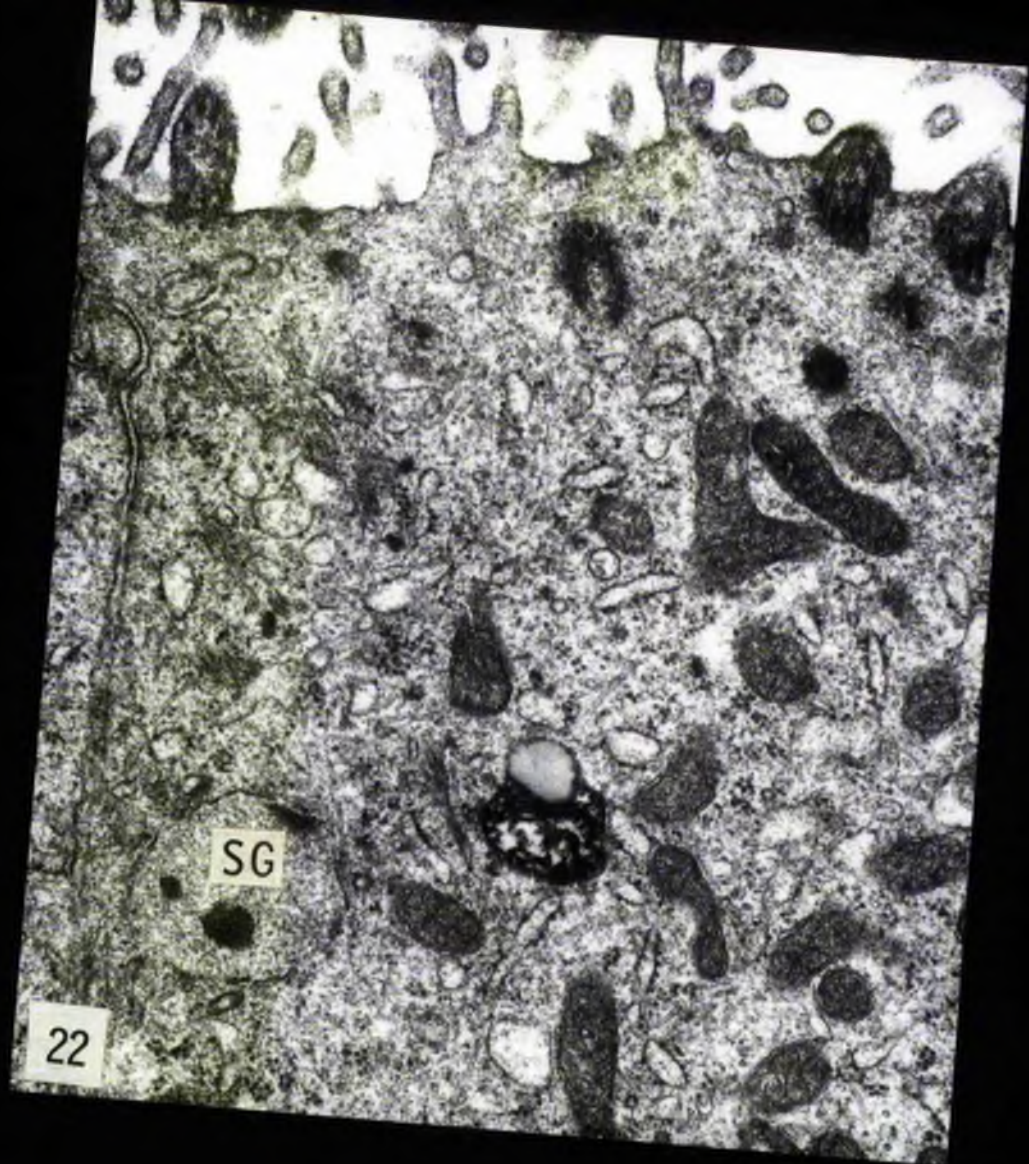


Figure 24: Epithelial basal lamina (BL).

a. Rat oviduct. UA&LC. X40,000

b. Rabbit oviduct. UA&LC. X40,000



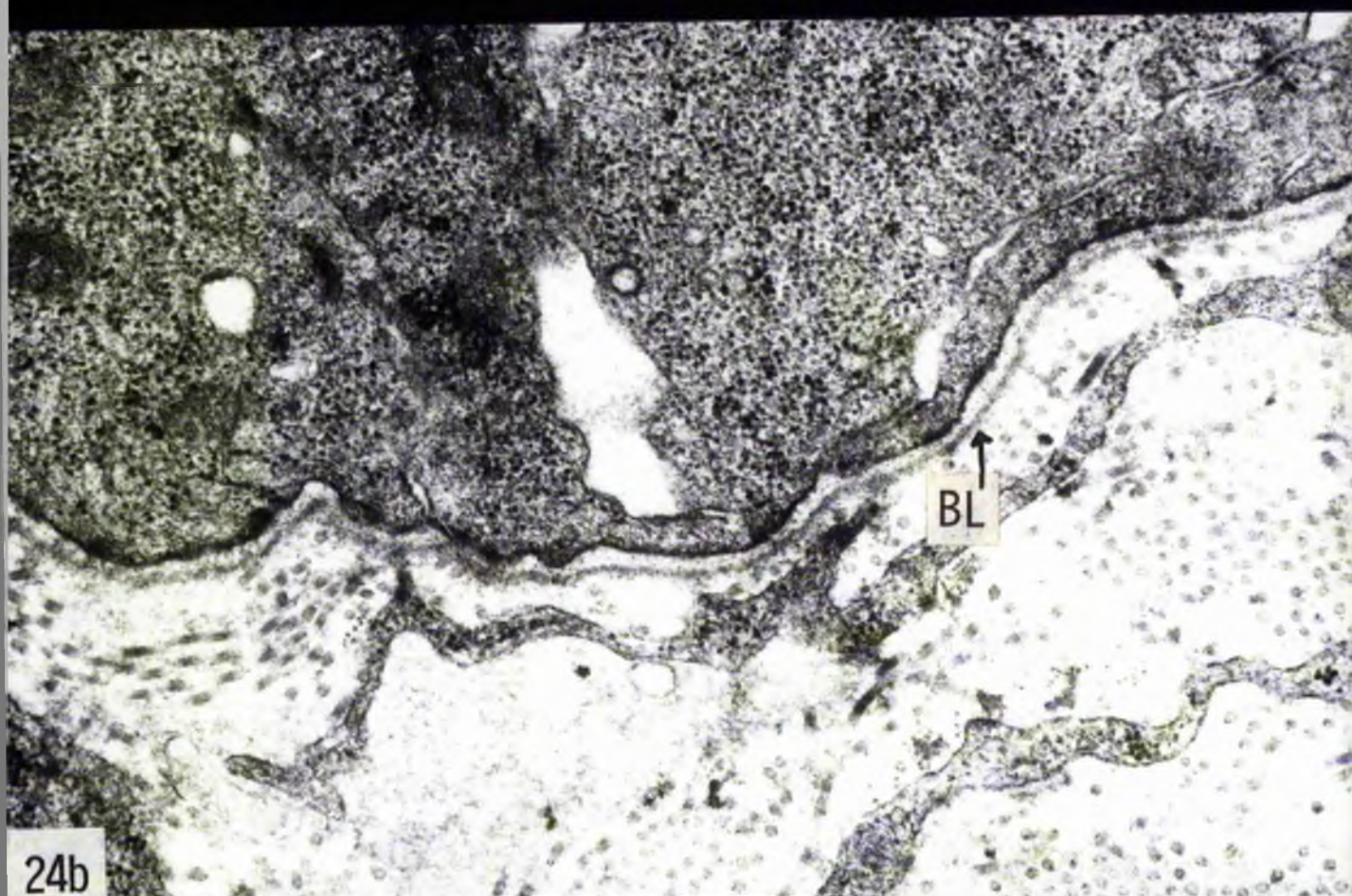
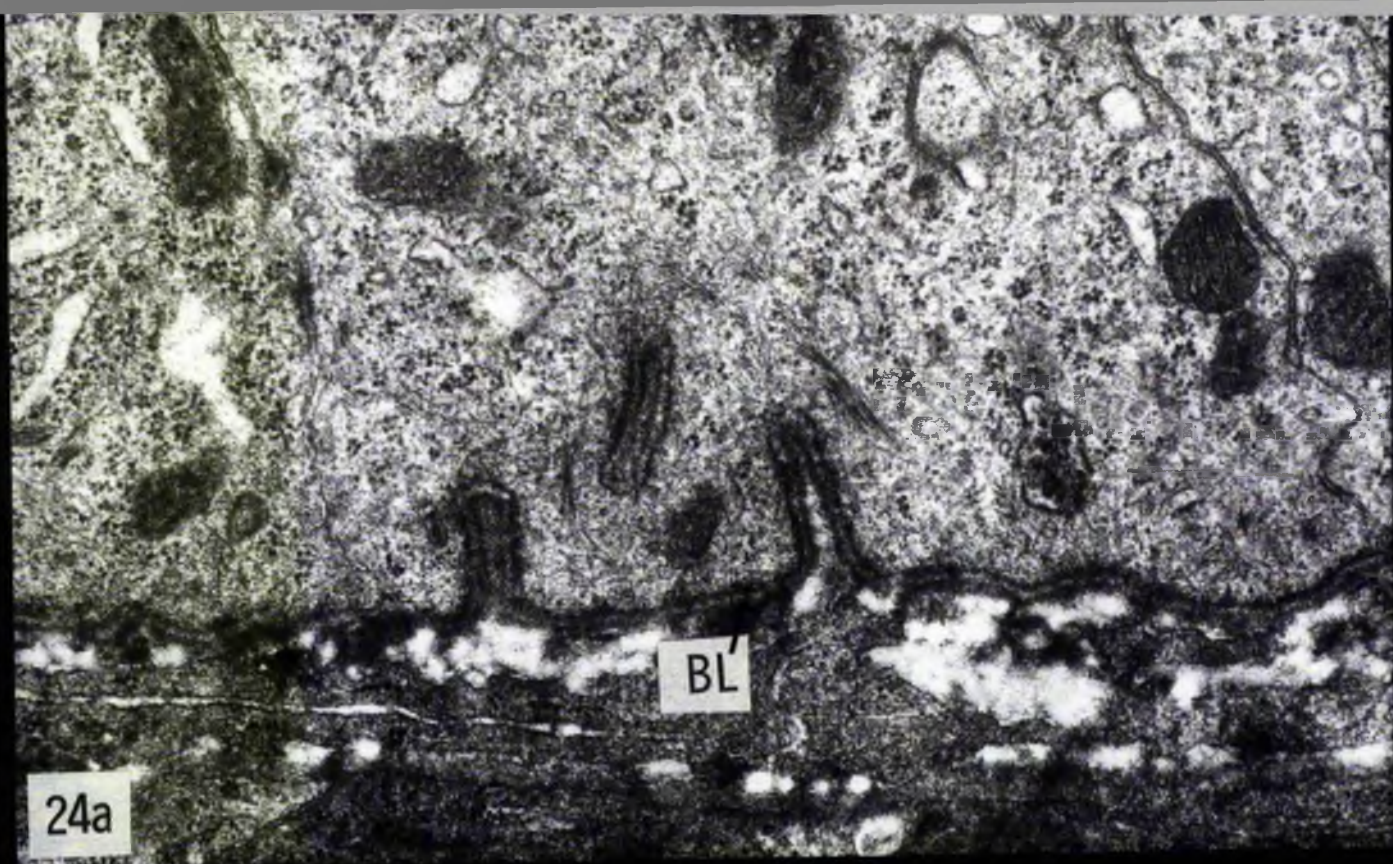




Figure 25: Macrophages in the intercellular space of the oviductal epithelium.

a. Rabbit oviduct. UA&LC. X27,615

BL- basal lamina

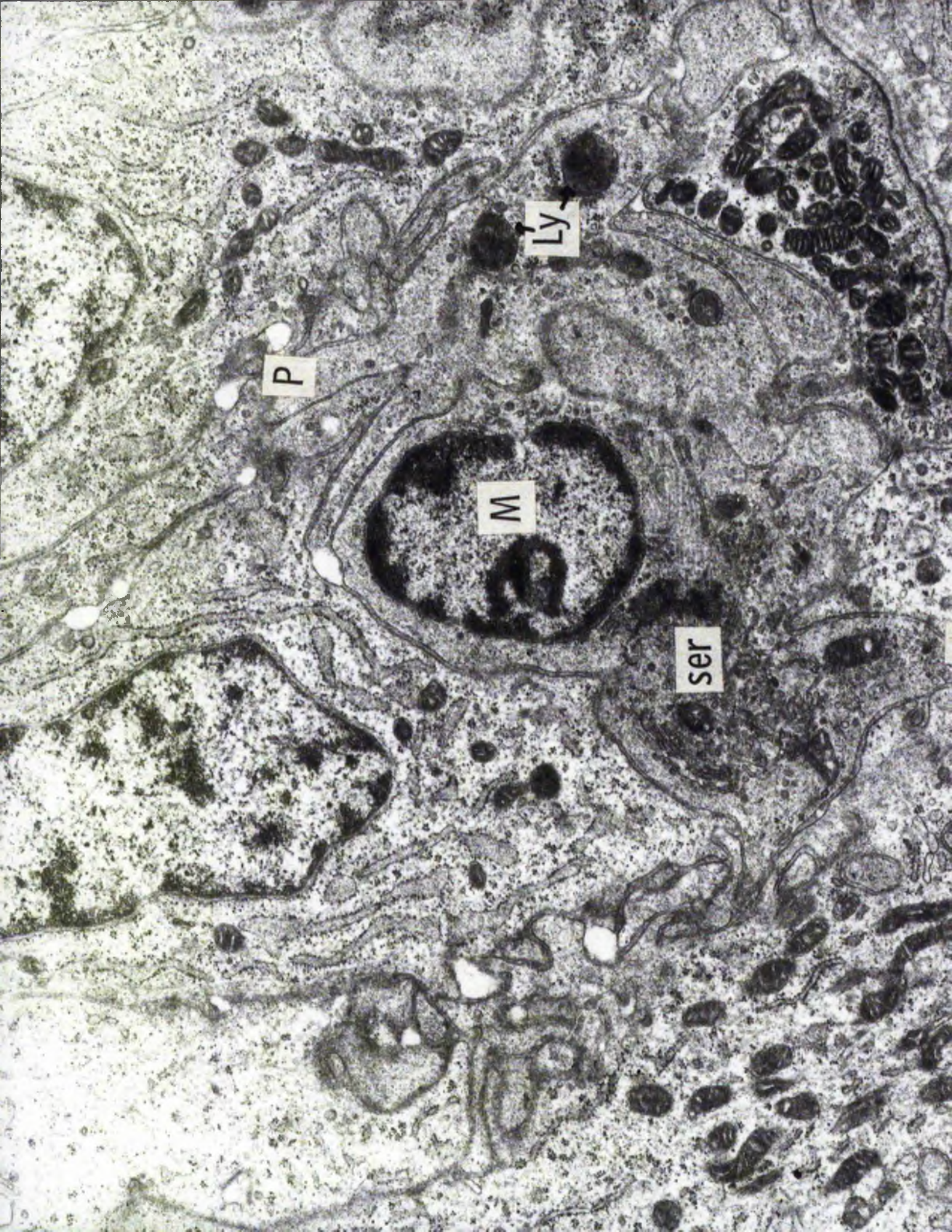
Ly- lysosomes

M- macrophage

P- pseudopodium

ser- smooth endoplasmic reticulum





Ly

P

M

ser



Figure 25 (continued):

b. Rabbit oviduct. UA&LC. X6510

BL- basal lamina

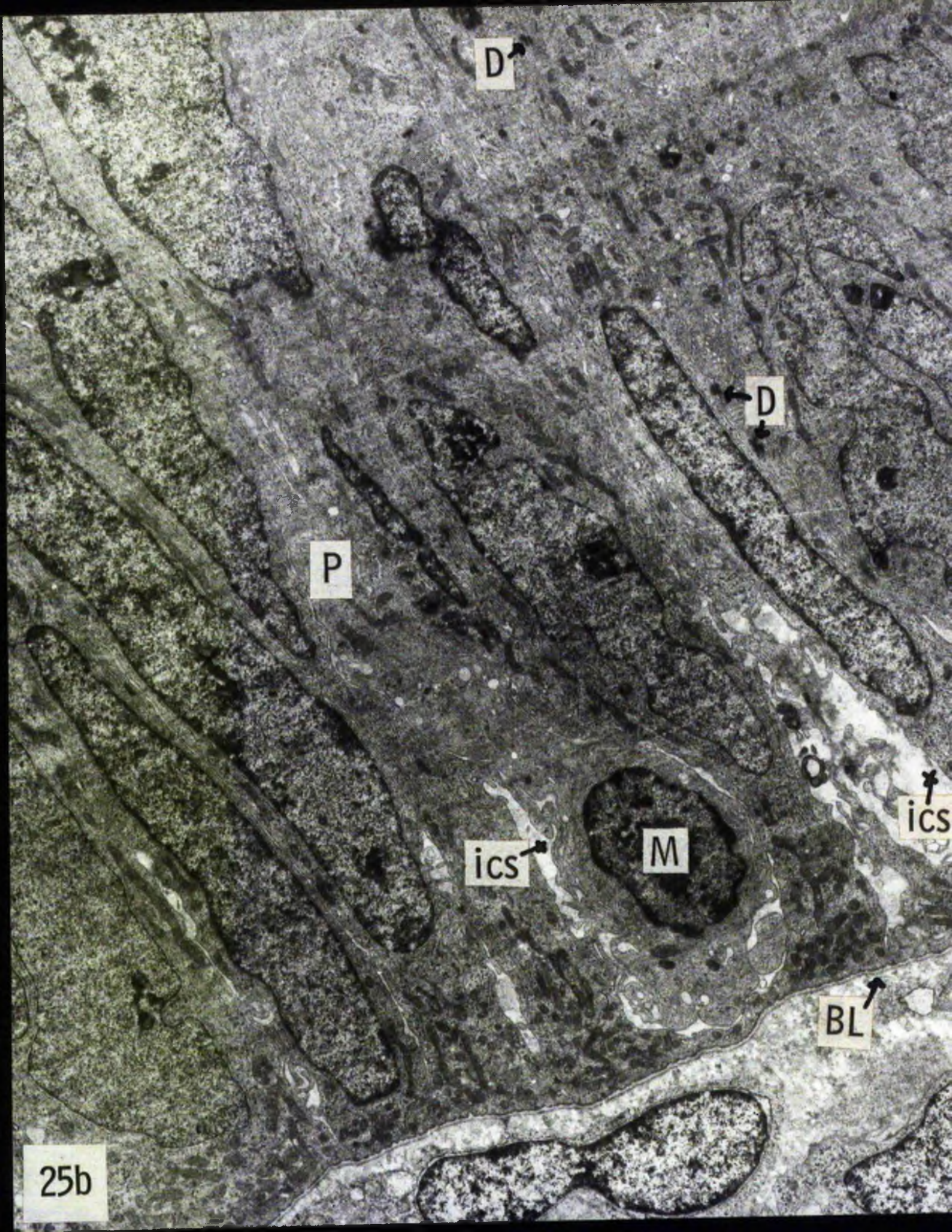
D- desmosomes between epithelial cells

ics- intercellular space

P- pseudopodia

M- macrophage







## II. THE EFFECTS OF SHORT-TERM EXOGENOUS ESTRADIOL ON THE EPI- THELIUM OF THE IMMATURE RAT AND RABBIT OVIDUCTS

### Oviductal Epithelium of the Immature Rat

Examination of the oviduct of the three-week-old rat pup shows that the epithelium already possesses ciliated cells and granulated secretory cells. Other cells are present which contain a small Golgi apparatus and a few segments of rough endoplasmic reticulum. A few 'granules' of varying densities can often be detected at the apices of these cells (Figs. 30, 31 & 34). These 'granules' are much smaller than those found in the differentiated secretory cells (see Figs. 5b & 33) and do not have the characteristic light and dark patches.

Some of the cells containing small granules also contain proliferative elements (Fig. 34). Cells in more advanced stages of ciliogenesis do not contain any apical granules, and therefore it would seem that the cells with the small apical granules are undifferentiated cells. The presence of these cells in the three-week-old rat, plus the appearance of cells in varying stages of ciliogenesis and the occasional mitosis indicates that at this age the oviductal epithelium is still undergoing proliferation and differentiation.

### Effect of Estradiol Treatment on Cell Volume, Nuclear Volume, Nucleo- cytoplasmic Ratio and Nucleolar Diameter of the Epithelial Cells of the Immature Rat Oviduct

The oviducts from the three rats receiving injections of estradiol for one, two and four days all showed significant ( $p < 0.01$  by



t-test) increases in cell and nuclear volume of the epithelial cells. The rest of the experimental rats however did not show differences in these parameters significant from control values (Table I, Figs. 26 & 27). No changes in nucleo-cytoplasmic ratio were observed in any of the oviducts of estradiol-treated rats.

The nucleoli of the epithelial cells were significantly ( $p < 0.01$  by t-test) larger in the oviducts of rats treated with estradiol for one, two, three and four days when compared to the controls (Table I, Fig. 28). Following five and six days of estrogen treatment, however, the nucleoli had returned to within control values.

All of the experimental rats showed an increase in uterine weight following estradiol treatment (Fig. 29). The uterine horns of the rat treated for three days were considerably distended with fluid.

#### Effect of Estradiol Treatment on the Ultrastructure of the Immature Rat's Oviductal Epithelium

It was difficult to discern any noticeable changes in the oviductal epithelium of the immature rats during the first few days of estrogen treatment. Following two estrogen injections the rough endoplasmic reticulum of the oviductal cells appeared to be somewhat more dilated with a flocculent electron-lucent substance, when compared with the controls. There also appeared to be an increase in the amount of rough endoplasmic reticulum present within the cells, especially in the basal regions of the cytoplasm (Fig. 31).

The distension of rough endoplasmic reticulum was particularly marked after five and six estradiol injections (Figs. 32 & 33). At the light microscope level, the isthmus of the oviduct of the rat

Table I: Effect of Estrogen Treatment on Cell Volume( $\mu^3$ ), Nuclear Volume( $\mu^3$ ), Nucleo-cytoplasmic Ratio (%) and Nucleolar Diameter( $\mu$ )

TREATMENT (1 $\mu$ g estradiol-benzoate in 0.1ml oil/day)			MEAN $\pm$ S.E.	
(Figures in parentheses indicate sample number)				
Controls (no estrogen treatment)				
Cell Volume	(35)	595.244 $\pm$	56.178	
Nuclear Volume	(35)	129.109 $\pm$	10.906	
Nucleo-cytoplasmic Ratio	(35)	22.691 $\pm$	1.133	
Nucleolar Diameter	(60)	1.149 $\pm$	0.033	
One Day Estrogen				
Cell Volume	(18)	968.763 $\pm$	69.994**	
Nuclear Volume	(18)	188.132 $\pm$	11.817*	
Nucleo-cytoplasmic Ratio	(18)	19.967 $\pm$	1.060	
Nucleolar Diameter	(30)	1.331 $\pm$	0.065*	
Two Days Estrogen				
Cell Volume	(20)	1011.939 $\pm$	99.366**	
Nuclear Volume	(20)	241.084 $\pm$	23.596**	
Nucleo-cytoplasmic Ratio	(20)	26.560 $\pm$	3.791	
Nucleolar Diameter	(30)	1.459 $\pm$	0.074**	
Three Days Estrogen				
Cell Volume	(17)	740.746 $\pm$	50.359	
Nuclear Volume	(17)	153.185 $\pm$	10.110	
Nucleo-cytoplasmic Ratio	(17)	21.429 $\pm$	1.280	
Nucleolar Diameter	(30)	1.376 $\pm$	0.052**	
Four Days Estrogen				
Cell Volume	(13)	1176.008 $\pm$	114.294**	
Nuclear Volume	(13)	226.743 $\pm$	18.204**	
Nucleo-cytoplasmic Ratio	(13)	18.724 $\pm$	1.200	
Nucleolar Diameter	(30)	1.373 $\pm$	0.059**	
Five Days Estrogen				
Cell Volume	(14)	784.378 $\pm$	36.144	
Nuclear Volume	(14)	156.327 $\pm$	16.924	
Nucleo-cytoplasmic Ratio	(14)	19.710 $\pm$	1.650	
Nucleolar Diameter	(30)	1.243 $\pm$	0.059	
Six Days Estrogen				
Cell Volume	(19)	708.240 $\pm$	37.093	
Nuclear Volume	(19)	154.519 $\pm$	13.287	
Nucleo-cytoplasmic Ratio	(14)	22.407 $\pm$	1.939	
Nucleolar Diameter	(30)	1.187 $\pm$	0.039	

\* Significantly higher than control,  $p < 0.01$  by t-test.

\*\* Significantly higher than control,  $p < 0.001$  by t-test.

which had received estrogen for six days appeared to contain several large intraepithelial 'cysts'. Examination of these 'cysts' with the electron microscope revealed them in fact to be intracellular microvillous vesicles. An electron-lucent substance which resembled the material found in the distended segments of rough endoplasmic reticulum was present within the vesicles (Fig. 33-MV).

These vesicles in many ways resemble ciliary vacuoles. The way that the cell nucleus is related to the microvillous vesicle is very reminiscent of the half-moon shape adapted by nuclei in many of the cells containing ciliary vacuoles (Fig. 33 c.f. Fig. 18a). Most of the rat oviducts exposed to estradiol for four weeks or longer (Part III) also contained microvillous vesicles, and an examination of many of these vesicles suggested that they were formed in a manner analogous to that described for the formation of ciliary vacuoles in Part I. That is, segments of rough endoplasmic reticulum, dilated with an electron-lucent substance, fuse together to form one large intracellular vesicle (see Fig. 50). At some stage during this process, the ribosomes are lost from the membranes of the endoplasmic reticulum. Eventually microvilli (or cilia, in the case of ciliary vacuoles) are formed, which project into the interior of the vesicle.

Only one other feature was noticed in the ultrastructure of the oviductal cells of the estrogen-treated rats. This was the appearance of nuclear inclusions in the epithelium of the rats treated with estrogen for four, five and six days. These inclusions (Figs. 32, 33 & 35) seem to be spherical or ovoid membranes, which in cross section appear as ring-like structures. Inclusions with

a more variable structure were also found (Fig. 35). Fine electron-dense particles can usually be detected inside the inclusions.

#### Oviductal Epithelium of the Immature Rabbit

Well differentiated ciliated and secretory cells are already present in the oviductal epithelium of the eight-week-old rabbit (Fig. 11a). However, the granules of the secretory cells of the immature oviduct are much smaller and fewer in number than those found in the mature oviduct (Fig. 11a compared to 11b). Although there is a considerable amount of rough endoplasmic reticulum present in the immature animal, the cisternae are relatively undistended (see Fig. 11a).

Undifferentiated columnar cells are also found, often exhibiting a solitary, rather rudimentary cilium with an accompanying centriole located subjacent to the ciliary basal body. These cells, and in addition, cells involved in ciliogenesis, indicate that at this stage the oviductal epithelium is still involved in the process of differentiation. Cells containing ciliary vacuoles are also seen in the rabbit at eight weeks (Fig. 18a).

#### The Effect of Ovariectomy on the Oviductal Epithelium of the Immature Rabbit

One week following ovariectomy, secretory cells no longer projected above the level of ciliated cells in the oviductal epithelium. Two weeks following ovariectomy there was a significant atrophy of both of the epithelial cell types (Table II, Figs. 36 & 40). Although there was a decrease in size of the ciliated cells, there was no loss of cilia at the time of the examination.

Table II: Effect of Estrogen Treatment on Cell Volume ( $\mu^3$ ), Nuclear Volume ( $\mu^3$ ), Nucleo-cytoplasmic Ratio (%) and Nucleolar Diameter ( $\mu$ )

TREATMENT (1  $\mu$ g estradiol-benzoate in 0.1ml oil/day) MEAN  $\pm$  S.E.  
(Figures in parentheses indicate sample number)

Controls (no estrogen treatment)

Cell Volume	(38)	789.165 $\pm$ 66.321
Nuclear Volume	(38)	192.635 $\pm$ 16.161
Nucleo-cytoplasmic ratio	(38)	26.904 $\pm$ 1.705
Nucleolar Diameter	(60)	0.970 $\pm$ 0.033

Ovariectomized Control

Cell Volume	(20)	445.842 $\pm$ 24.280
Nuclear Volume	(20)	165.437 $\pm$ 13.698
Nucleo-cytoplasmic Ratio	(20)	37.361 $\pm$ 2.653
Nucleolar Diameter	(21)	0.892 $\pm$ 0.041

One Day Estrogen (Litter 1)

Cell Volume	(16)	2531.589 $\pm$ 241.679
Nuclear Volume	(16)	420.471 $\pm$ 37.553
Nucleo-cytoplasmic Ratio	(16)	17.388 $\pm$ 1.402
Nucleolar Diameter	(26)	1.192 $\pm$ 0.044

Two Days Estrogen (Litter 1)

Cell Volume	(15)	1887.429 $\pm$ 194.024
Nuclear Volume	(15)	298.107 $\pm$ 34.418
Nucleo-cytoplasmic Ratio	(15)	16.633 $\pm$ 1.411
Nucleolar Diameter	(18)	1.169 $\pm$ 0.038

Three Days Estrogen (Litter 2)

Cell Volume	(12)	1508.414 $\pm$ 179.434
Nuclear Volume	(12)	301.224 $\pm$ 35.642
Nucleo-cytoplasmic Ratio	(12)	21.072 $\pm$ 2.458
Nucleolar Diameter	(30)	1.463 $\pm$ 0.061

Four Days Estrogen (Litter 2)

Cell Volume	(18)	1992.896 $\pm$ 281.63
Nuclear Volume	(18)	329.765 $\pm$ 33.76
Nucleo-cytoplasmic Ratio	(18)	18.674 $\pm$ 1.90
Nucleolar Diameter	(18)	0.896 $\pm$ 0.03

Three Days Estrogen (Litter 3)

Cell Volume	(20)	2071.483 $\pm$ 153.264
Nuclear Volume	(20)	375.043 $\pm$ 29.519
Nucleo-cytoplasmic Ratio	(20)	18.504 $\pm$ 1.054
Nucleolar Diameter	(39)	1.048 $\pm$ 0.041



Table II (Continued)

TREATMENT (1 $\mu$ g estradiol-benzoate in 0.1ml oil/day)		MEAN $\pm$ S.E.
Four Days Estrogen (Litter 3)		
Cell Volume	(18)	1106.528 $\pm$ 114.851
Nuclear Volume	(18)	222.545 $\pm$ 33.051
Nucleo-cytoplasmic Ratio	(18)	20.897 $\pm$ 1.933
Nucleolar Diameter	(26)	0.981 $\pm$ 0.032
Five Days Estrogen (Litter 3)		
Cell Volume	(17)	1497.392 $\pm$ 145.192
Nuclear Volume	(17)	323.999 $\pm$ 27.781
Nucleo-cytoplasmic Ratio	(17)	22.391 $\pm$ 1.220
Nucleolar Diameter	(30)	0.946 $\pm$ 0.027
Six Days Estrogen (Litter 3)		
Cell Volume	(18)	1041.561 $\pm$ 140.850
Nuclear Volume	(18)	216.769 $\pm$ 29.795
Nucleo-cytoplasmic Ratio	(18)	21.016 $\pm$ 1.487
Nucleolar Diameter	(30)	0.994 $\pm$ 0.040

\* Significantly higher than control,  $p < 0.05$  by t-test.

\*\* Significantly higher than control,  $p < 0.01$  by t-test.

\*\*\* Significantly higher than control,  $p < 0.001$  by t-test.

△ Significantly lower than intact control,  $p < 0.001$  by t-test.

The nonciliated cells, on the other hand, appeared to be more sensitive to the effects of hormone deprivation. A considerable decrease in cytoplasmic volume of these cells, especially in the ampullar portion of the oviduct, resulted in the formation of cone-shaped elements with a high nucleo-cytoplasmic ratio and containing few signs of active protein synthesis (Fig. 40).

The Effect of Estradiol Treatment on Cell Volume, Nuclear Volume, Nucleo-cytoplasmic Ratio and Nucleolar Diameter of the Epithelial Cells of the Immature Rabbit Oviduct

The average oviductal epithelial cell volume of all of the estrogen-treated immature rabbits was greater than the average cell volume of the controls, and in only one instance was the difference not significant. A significantly greater average nuclear volume was also observed in six of the estrogen-treated animals (Table II, Figs. 36 & 37). No significant differences compared to the controls were observed in the nucleo-cytoplasmic ratios of any of the experimental rabbits.

Only two experimental rabbits exhibited significant increases in nucleolar diameter (Table II, Fig. 38). These were the two rabbits in litter one which had received estradiol for one and two days. The average nucleolar diameter of the rabbit in litter two which had received estradiol for three days was also significantly higher than the average found for the controls. However ultrastructure examination of the oviductal epithelium of this rabbit revealed improper fixation, with 'edema' of the nuclei and distortion of the nucleolar material (Fig. 41). Therefore the high average nucleolar diameter in the oviduct of this animal was considered

to be artifactual.

The Effect of Estradiol Treatment on the Ultrastructure of the  
Immature Rabbit's Oviductal Epithelium

Electron microscopic examination of the oviductal epithelial cells of the estradiol-treated immature rabbits revealed striking alterations in only one animal. The nonciliated cells of the rabbit in litter one which had received estradiol for two days contained large amounts of rough endoplasmic reticulum which was in many situations greatly distended with an electron-lucent substance (Fig. 42). Clear cut evidence of increased synthetic activity was not observed in any of the other oviducts.

Effect of Estradiol Treatment on Uterine Weight and Ovarian Histology  
of the Immature Rabbit

The effect of the estradiol treatment on the uterine weight of the immature rabbits is illustrated in Figure 39. As can be seen from this graph, only three of the experimental rabbits responded to the estrogen injections with increases in uterine weight. None of the four rabbits in litter three experienced an increase in uterine weight, and the uterus of only one of the two experimental rabbits in litter two responded to the hormone. However both of the experimental rabbits in litter one responded to the estrogen injections with weight increases in their uteri, and the uterus of the rabbit receiving two injections of estrogen weighed approximately one and a half times as much as the rabbit receiving only one estrogen injection.

The ovaries of all of the rabbits were studied with the light microscope. This examination revealed that not all of the animals were at the same stage as regards ovarian development (Table III). The ovaries of five of the rabbits were immature in the sense that they only contained primordial follicles. Other rabbits had growing follicles and an occasional mature Graafian follicle in their ovaries. And finally, four of the rabbits' ovaries contained many Graafian follicles and were indistinguishable from those of the fourteen-week-old rabbits examined in Part I. None of the ovaries of the control or experimental rabbits contained corpora lutea.

An attempt to correlate ovarian histology with estrogen treatment proved inconclusive. There was also no apparent relationship between ovarian size or histology and uterine weight.

Although the ovaries of the rabbit in litter two which had been ovariectomized contained only primordial follicles, there was still considerable atrophy in the oviductal epithelium two weeks after the operation. This indicated that the level of oviductal epithelial cell growth that was found in the eight-week-old control rabbits was dependent on hormonal stimulation from the animals' ovaries.

Table III: Effect of Exogenous Estradiol on Ovarian Histology  
and Ovarian and Uterine Weight of the Immature Rabbit

Animal	Ovarian Histology	Ovarian Weight (both ovaries)	Uterine Weight
E1/1 (control)	Many Graafian follicles; no corpora lutea.	94mg	200mg
E1/2 (ovariect. one week prior to sacrifice)	Many Graafian follicles; no corpora lutea.		178mg
E1/4 (one day estrogen)	Many Graafian follicles; no corpora lutea.	83mg	918mg
E1/3 (two days estrogen)	Three Graafian follicles per section; no c. lutea.	60mg	1572mg
E2/1 (control)	Primordial follicles only; no corpora lutea.	49mg	172mg
E2/2 (ovariect. two weeks prior to sacrifice)	Primordial follicles only; no corpora lutea.	44mg	146mg
E2/4 (three days estrogen)	Primordial follicles only; no corpora lutea.	33mg	300mg
E2/3 (four days estrogen)	Many Graafian follicles; no corpora lutea.	99mg	1817mg



Table III(continued)

Animal	Ovarian Histology	Ovarian Weight (both ovaries)	Uterine Weight
E3/1 (three days estrogen)	Primordial and growing follicles; no c. lutea.	28mg	140mg
E3/2 (four days estrogen)	One Graafian follicle per section; no c. lutea.	42mg	315mg
E3/3 (five days estrogen)	Primordial follicles only; no corpora lutea.	44mg	192mg
E3 /4 (six days estrogen)	Primordial follicles only; no corpora lutea.	33mg	346mg

THE EFFECTS OF SHORT-TERM EXOGENOUS ESTRADIOL ON THE EPITHELIUM  
OF THE IMMATURE RAT AND RABBIT OVIDUCTS: DISCUSSION

Cytodifferentiation of the Oviductal Epithelium

Cytodifferentiation of the oviductal epithelium can be considered to be the appearance of cells that are morphologically indistinguishable from those found in the adult oviduct. In the rat and the rabbit, as in most mammalian species, differentiation is marked by ciliogenesis and the development of cells adapted to secretory granule formation. The process of differentiation starts during the first postnatal week in rats and rabbits and continues for several weeks thereafter (Kellogg, 1945; McCarron and Anderson, 1973).

Kellogg (1945) noticed that the apices of the undifferentiated cells lining the neonatal rat oviduct contained a very small amount of mucicarmine-positive material, which also faintly stained the lumen of the oviduct. Ciliogenic cells containing basal bodies however had lost all traces of the mucoid material.

The cells observed in the present study which possessed small homogeneous granules probably correspond to the undifferentiated mucin-containing cells described by Kellogg. Proliferative elements were occasionally observed in these undifferentiated granule-containing cells. However by the time that generative complexes and mature centrioles appeared in the cytoplasm the granules had disappeared. It is interesting to note that Kalnius and Porter (1969) also observed 'mucus droplets' in the apical cytoplasm of cells during early ciliogenesis in the tracheal epithelium of embryonic chicks. The droplets were absent in cells containing more mature centrioles. They concluded that the tracheal epithelial cells

differentiated for mucus secretion at an early stage in development, and it wasn't until a later stage in differentiation that ciliogenesis occurred.

The undifferentiated cells of the immature rabbit oviduct often displayed a single cilium on the luminal surface. McCarron and Anderson (1973) discuss the significance of these single cilia and their probable origin from the cell's diplosomal centrioles, and review other instances where single cilia are observed during development of a tissue.

#### Estrogen and Induction of Cytodifferentiation in the Oviductal Epithelium

The results of experiments using several animal species have implicated estrogen as the inducing stimulus for oviductal epithelial differentiation (see Introduction, p21 ). In the present study, differentiation was well under way in the immature rat and rabbit oviductal epithelium by the time that estrogen treatment had begun. The qualitative morphological study did not reveal any alterations in the differentiation process as a result of the estrogen treatment.

Nevertheless, some discussion of the evidence concerning the possible involvement of estrogen in the regulation of epithelial cytodifferentiation in the oviducts of the rat and rabbit is in order. Some of the effects on the reproductive tract of the former species of long-term exposure to estrogen (Part III) further validate such a discussion.

So far there is no evidence that estrogen induces ciliogenesis in the undifferentiated rat oviduct. In fact, there is evidence

against such a supposition. Dubuissou et al. (1972) found that ciliated cells appeared in the oviducts of rats ovariectomized on the day of birth at the same time as they appeared in the controls (i.e. six days after birth).

Weisz and Gunsalus (1973) detected estradiol in the blood of female rats using radioimmunoassay (RIA) during the first postnatal week, which from their data, they deduced was of adrenal origin. This could mean that in the experiments of Dubuissou et al., the ovariectomized neonatal rats' adrenals were providing the estrogen necessary to induce ciliation. However the extremely high levels of estradiol and estrone detected using RIA by Weisz and Gunsalus in the neonatal rats caused these authors to doubt the veracity of their assay.

In an earlier study, Presl, Herzmann and Horský (1969) calculated the estrogen concentration in the blood of developing rats using a fluorimetric assay. They found that the level of fluorogens detected in the blood of five-day-old female rats did not differ from those found in ovariectomized-adrenalectomized adult female rats or in normal males, and therefore concluded that the five-day-old level could be attributed to trace impurities. An increase in blood estrogen levels (i.e. from the baseline attributed to impurities) was only detected at ten days after birth. This coincided more or less with the appearance of steroid-3 $\beta$ -ol dehydrogenase (an enzyme involved in estrogen synthesis) in the immature rats' ovaries (Presl et al., 1965).

If the results of the study by Presl and his colleagues are taken to be more accurate than those of Weisz and Gunsalus, then it would appear that ciliogenesis occurs in the rat oviduct prior

to the time when estrogen can be detected in the blood. Dubuisson et al. (1972) did not indicate whether neonatal ovariectomy had any effect on the subsequent development of secretory cells in the rat oviduct. Although detecting ciliogenic cells in the oviduct during the first postnatal week, Kellogg (1945) did not observe an increase in mucus production from the low level attributed to the undifferentiated cells until the third week after birth. At this time the mucicarmine staining of the oviductal lumen intensifies and mucus granules begin to accumulate gradually in the nonciliated cell apices.

The sequence of biochemical events initiated in target cells by estrogen has been outlined in the Introduction (p 10). In the rat uterus the 'specific response' which follows the intracellular binding of estradiol to its receptor molecule involves the synthesis of 'induced protein' and the mRNA responsible for that protein's synthesis. This is followed by an increase in nuclear RNA synthesis and subsequently, cytoplasmic protein synthesis.

Sömjen and her colleagues (Sömjen et al., 1973) have studied the postnatal development of this response in the rat uterus and have discovered that the capacity for a full response is acquired only gradually. Although cytoplasmic and nuclear binding sites for estradiol are present in the uterus on the day of birth, 'induced protein' is not synthesized in response to estrogenic stimulation until the fifth postnatal day. An increase in cellular protein synthesis cannot be provoked by the hormone until ten to fifteen days after birth.

If one assumes that the ability to respond to estrogenic stimulation develops in the rat oviduct at the same time as it



does in the uterus, then ciliation occurs prior to the maturation of the 'specific response'. However, increase in mucus production, i.e. differentiation of oviductal secretory cells, occurs after the cells have fully developed the capacity to respond to estrogen. All of the evidence produced so far implies that ciliogenesis in the undifferentiated rat oviductal epithelium is outwith the control of estrogen, whereas differentiation of the mucus producing cells of the oviduct may be induced by this hormone.

McCarron and Anderson (1973) also observed a slight delay between the initiation of ciliogenesis and the initiation of secretory cell differentiation in the immature rabbit oviduct. Neonatal ovariectomy of the rabbit, as in the rat, has no effect on the subsequent development of ciliated cells (Hafez and Kodituwakku, 1970). However 180 days after neonatal ovariectomy the height of the epithelium was considerably reduced and the motility of the cilia was feeble when compared to controls. Again, no comment was made concerning the effects of the neonatal ovariectomy on the subsequent development of secretory cells in the rabbit oviduct.

Although neonatal ovariectomy does not prevent the appearance of ciliated cells in the rabbit oviduct, the same operation performed on the adult eventually results in widespread loss of cilia (Flerko, cited in Brenner, 1969a; Rumery and Eddy, 1974). Ciliation is restored in the ovariectomized adult rabbit oviduct following estrogen treatment (Ibid.) and as Dubuisson et al. (1972) found no loss of cilia even six months after ovariectomy of the adult rat, it seems likely that the control of ciliogenesis in these two species may be different. Until further evidence is produced however, no concrete conclusions regarding the rôle of hormones

in the postnatal cytodifferentiation of the oviducts of either the rat or rabbit can be drawn.

Many early studies reported an accelerated development of the Müllerian duct following the injection of estrogen into mammalian and bird female embryos (Burns, 1961). Estrogen administration will stimulate ornithine decarboxylase activity in the undifferentiated chick oviduct (O'Malley et al., 1974) and this enzyme has been linked with the chemical induction of growth in a variety of cell types. Sömjen et al. (1973) found that an increase in ornithine decarboxylase synthesis could be induced by estradiol in the uteri of two to five-day-old rats, i.e. before the maturation of the 'specific response' to estradiol had occurred. This evidence suggests that the ability to respond to estradiol stimulation with increased ornithine decarboxylase synthesis (and possibly increased growth) may be a property of undifferentiated cells of the reproductive tract. The hypertrophy reported in the Müllerian duct following estrogen administration in these early experiments does not necessarily mean therefore that the reproductive tract was undergoing a precocious development.

#### Effects of Estrogen Administration on the Oviductal Epithelium of the Immature Rat

i. Cell, Nuclear and Nucleolar Size. Although exogenous estrogen did not appear to have any effect on the course of differentiation observed in the oviduct of the immature rat, it did cause an increase in cell and nuclear volume of the epithelial cells. Average values for cell and nuclear volumes had risen signif-

icantly twenty-four hours after a single injection of estradiol-benzoate, and remained high after two and four days of estrogen treatment. (At this point, no explanation can be found for the lack of significant differences in cell and nuclear volume observed between the rat treated for three days with estrogen and the control values.)

Estradiol injections also effected a significant increase in the nucleolar diameter of the oviductal epithelial cells. The increase in nucleolar size was observed during the first four days of estrogen treatment.

These findings are consistent with the results obtained by other workers who investigated the morphological alterations in a variety of target tissues in response to estradiol stimulation. Tachi, Tachi and Linder (1974) found an increase in nuclear and cytoplasmic volume of uterine stromal cells twelve hours after estrogen administration to ovariectomized, adrenalectomized rats. They also found an increase in nucleolar size in both the luminal and glandular epithelial cells of the rat uterus that was first noticeable five hours after estrogen administration, and which became quite striking at twelve hours (Tachi, Tachi and Linder, 1972). Williams and Rodgers (1972) also found increases in the nuclear and cytoplasmic volumes of luminal epithelial cells of the ovariectomized rat uterus twenty-five hours following estrogen treatment. Laguens (1964) found that the nucleoli of rat uterine smooth muscle cells remained larger than those of the controls even three days after a single subcutaneous injection of 10µg estradiol monobenzoate. (This is probably due to the fact that esters of estradiol administered subcutaneously are absorbed gradually, thus prolonging

the actual period of estrogenic stimulation.)

All of the preceeding experiments, however, were performed on ovariectomized (and in some cases, adrenalectomized) adult rats, whose reproductive organs prior to treatment would have atrophied as a result of endogenous hormone withdrawal. Only a few similar experiments have been performed on immature animals whose ovaries are already producing small, but significant amounts of estrogen.

Kang, Anderson and DeSombre (1975) examined the effect of estradiol-17 $\beta$  on uterine growth and morphology of immature rats of the same age and strain as those examined in the present study (i.e. Sprague-Dawley rats of 21-23 days of age). They divided the uterine mucosa into three regions, starting at the oviductal end of the uterine horn and ending at the cervix (i.e. post-oviduct, corpus and adcervical). Estrogen injections were given daily (0.1-0.4 $\mu$ g estradiol-17 $\beta$  administered subcutaneously in 0.1ml glycerol), and Kang and his colleagues found that the responses of the epithelium in the three regions differed.

All of the regions showed an increase in epithelial cell height, however the epithelium in the adcervical region showed the greatest response (54% increase over control height, twenty-four hours after the first injection). The epithelial cells in the postoviductal region of the uterus showed an increase in height of 33% over the controls twenty-four hours after the first injection. The cells of the corpus and adcervical regions continued to increase in height with further estrogen treatment and following three days showed an 68% and 118% increase respectively. However, following three daily injections the height of the epithelial cells in the

postoviductal region showed great variation, and only averaged an 8% increase over the control height. This is particularly interesting in view of the lack of significant differences between the cell and nuclear volumes of the oviductal cells of the control and experimental rat treated with estrogen for three days in the present study.

ii. Protein Synthesis. On a purely subjective basis there appeared to be an increase in the amount and dilation of rough endoplasmic reticulum (RER) in the nonciliated cells of the immature rat oviduct following estrogenic stimulation. It was difficult to detect this increase after one day of estrogen treatment, because RER and secretory granules were already present in many of the nonciliated cells of the 'unstimulated' immature oviduct. However after several days of estrogen treatment there was a clear amplification in the level of protein synthesis observed in the experimental oviducts when compared to the low level observed in the controls.

Many investigators have found morphological evidence for an increase in protein synthesis following estrogenic stimulation of a target tissue. William and Rodgers (1972) found an increase in the amount of RER present within the uterine luminal epithelial cells of the ovariectomized rat twenty-five hours after estrogen administration. Bo, Odor and Rothrock (1968) found a gradual increase in the amount and dilation of the RER of rat uterine smooth muscle cells from six to ninety-six hours after a single subcutaneous injection of 10µg estradiol dipropionate. And Brinsfield and Hawk (1974) observed a very pronounced prolifera-



tion of RER which was extensively dilated in the endometrial stromal cells of ovariectomized ewes following five days of estrogen treatment.

Again, all of the above studies were performed on ovariectomized animals and in the reproductive organs of these animals, protein synthesis has declined to a low baseline level. However Ross and Klebanoff (1967) did examine the effects of estrogen on protein synthesis in the uterine smooth muscle cells and fibroblasts of prepubertal rats (57-70g). They detected an increase in the numbers of free and attached ribosomes, and in the amount and dilation of RER following hormone treatment for three days.

If a technique such as that of linear analysis which was employed recently by Brinsfield and Hawk (1974) had been applied in the present instance, it is possible that the changes in RER content could have been quantified. However on the basis of the subjective observations made in this study, it can only be concluded that the 1µg daily injections of estradiol-benzoate resulted in a gradual increase in the level of protein synthesis which was observed in the untreated immature rat oviduct.

In the preceding discussion, no mention has been made of the lack of significant differences observed between the cell, nuclear and nucleolar sizes of the control oviducts and those of the rats receiving estrogen for five and six days. Nor was any reference made to the appearance of intraepithelial 'cysts' in the oviduct following six days of estrogen treatment. The interpretation of these findings was hindered until it was realized that they might have resulted from the effects of estrogenic stimulation of the

immature rat's hypothalamico-pituitary-ovarian axis, rather than the results of direct stimulation of the oviduct by estrogen.

#### The Hypothalamico-Pituitary-Ovarian Axis of the Immature Rat

The ovaries of the immature rat are secreting a small but significant amount of estradiol which acts as an inhibitor of both FSH and LH secretion by the pituitary. Ovariectomy of a rat at twenty-six days of age is followed by an immediate and sustained rise in both FSH and LH levels in the serum (McPherson et al., 1974).

Puberty in the rat is thought to be associated with a decrease in sensitivity of the hypothalamus to the inhibitory effects of ovarian estradiol on the release of gonadotropin-stimulating factors. Steele and Weisz (1974) tested this hypothesis by examining the dosage of estradiol necessary to suppress LH secretion in the ovariectomized pre- and post-pubertal rat. They found that a much smaller dosage of estradiol was needed to suppress LH release in the pre-pubertal rat, than was needed in the post-pubertal rat.

McPherson et al. (1974) also examined the role of ovarian estrogen in the negative feedback mechanism in the immature rat. They found that with the same dosage of estradiol there was a greater suppression of LH levels with FSH levels in the ovariectomized twenty-six-day-old rat. The amount of estradiol needed to suppress the gonadotropin secretion to the level found in the intact immature rat was quite small (i.e. 0.2 $\mu$ g/kg/day). At slightly higher dosages, serum gonadotropin levels began to increase, with a rise in FSH level beginning at the 0.8 $\mu$ g dosage and a rise in LH level beginning at the 1  $\mu$ g dosage level. This

indicates that at some dosage levels estrogen will stimulate, rather than inhibit, gonadotropin release in the immature rat. This also seems to indicate that a change in feedback sensitivity in the hypothalamus at puberty would lead to an increase in FSH levels prior to an increase in LH levels. The manner in which this might lead to ovarian maturation, ovulation and the onset of cyclicity is shown in Text-Figure II.

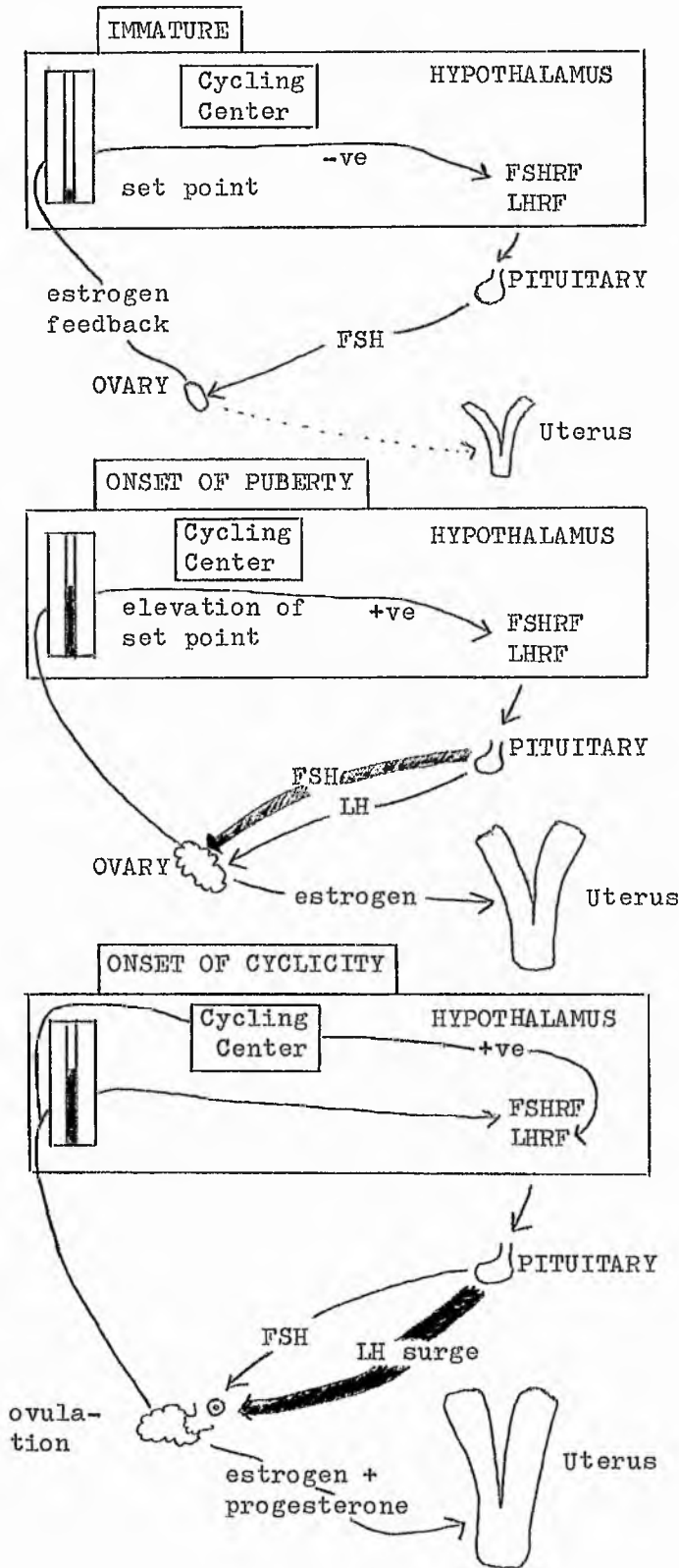
#### Effect of Exogenous Estrogen on the Hypothalamico-Pituitary-Ovarian Axis of the Immature Rat

A gradual increase in the amount of estrogen given to the ovariectomized immature rat first causes suppression, and then stimulation of gonadotropin release by the pituitary (McPherson et al., 1974). This suggests that rising levels of estrogen in the plasma caused either by an increase in endogenous output or an exogenous source, might actually provoke the change in the hypothalamic 'set point' (see Text-Fig. II). In the intact immature animal given estrogen the increased release of FSH causes follicle maturation, and as the level of estradiol rises even further, the subsequent release of LH can cause ovulation. This theoretical chain of events has been supported by experimental evidence.

The 'induction of puberty' by estrogen injection in the immature rat has been a long-recognized phenomenon. Using vaginal patency as a sign of maturity, Allen and Doisy (1924) induced puberty in immature rats with injections of 'ovarian follicular hormone'. Hohlweg subsequently demonstrated in 1934 (see Everett, 1961) that an injection of estrogen could induce ovulation and corpus

Text-Figure II: Postnatal Changes in the Hypothalamo-Pituitary-

Ovarian Axis (Slightly modified diagram from Ezrin, 1973)



The pituitary secretes a small amount of gonadotropin, chiefly FSH, and the ovary responds with a low level of estrogen release. The hypothalamic 'set point' is so low that the small amount of estrogen secreted by the ovary can exert negative feedback inhibition even though it exerts no 'demonstrable' biological effect. The hypothalamic cycling center is quiescent.

During puberty the 'set point' level for negative feedback inhibition progressively increases, thus gonadotropin output increases. As a result the estrogen levels rise and induce the appearance of the secondary sex characteristics. The cycling center is still dormant and therefore ovulation doesn't take place.

Eventually, the rising level of estrogen seems to exert a positive feedback effect on LHRF, and therefore triggers an LH surge that is sufficient to provoke ovulation. The awakening of the cycling center must occur before ovulatory cycles begin.

luteum formation. This experiment has been repeated by several workers (Döcke and Dörner, 1974; Mazer, Israel and Alpers, 1936; Price and Ortiz, 1944; Ramirez and Sawyer, 1965; Ying and Greep, 1971), but there appears to be differences in the age at which ovulation will occur in response to estradiol injection among the various rat strains.

It cannot be known for certain whether the estrogen treatment induced ovulation in the immature rats examined in the present study, as the ovaries were lost during the automatic processing procedure. However the ovaries of the rat treated for six days with estradiol did display two haemorrhagic follicles which were visible with the naked eye. This strongly suggests that ovulation was about to occur, or had already taken place, in this animal.

Nevertheless, there is other evidence that the ovaries of the rats treated with estradiol for four, five and six days were beginning to secrete progesterone and that this hormone was subsequently affecting the reproductive tract tissues.

A widely recognized phenomenon observed in the rat's uterus during the estrus cycle is the accumulation of intraluminal fluid on the day of proestrus and the subsequent release of the fluid into the vagina during estrus. The proestrus 'ballooning' of the uterine horns is caused by the increasing estrogen secretion by the rat's ovaries on the morning of proestrus (Swartz-see Armstrong, 1968a). The hormonal control mechanisms involved in uterine luminal fluid retention have been studied (Armstrong, 1968a&b; Kennedy and Armstrong, 1976) and the results of these studies have shown that the increasing secretion of progesterone on the afternoon of proestrus is essential for the eventual



relaxation of the cervixes.

Originally it was believed that the progesterone necessary for this release was secreted from the corpora lutea of the previous cycle in response to the LH surge on the afternoon of proestrus. However Armstrong (1968b) demonstrated in experiments using immature rats that had never ovulated that LH could also stimulate progesterone secretion from non-luteal ovarian elements.

The amount of progesterone needed to cause luminal fluid loss in the presence of estrogen (e.g. from hormonal implants or injections) is of such a low level that it does not inhibit the uterotrophic action of the estradiol. Because of its sensitivity to progesterone levels, Armstrong (1968a) believed that uterine fluid loss could be used as a semi-quantitative, physiological indicator of progesterone secretion.

In the present experiment it was observed that with daily estrogen treatment, uterine weight steadily increased until day three, when the uterine horns exhibited profound 'ballooning'. This is consistent with other studies using rats of the same strain and age (Kang, Anderson and DeSombre, 1975; Ross and Klebanoff, 1967). However estrogen treatment in these studies did not continue beyond three days. Using a different strain of rats (21-23 day Holtzmann), Armstrong (1968a) found release of luminal fluid 114 hours (4.6 days) after subcutaneous implantation of an estradiol pellet. This is in agreement with the present finding that the uteri of the rats examined after four days of estrogen treatment did not exhibit ballooning. This is also strong evidence that the ovaries of these rats were secreting enough progesterone to cause cervical relaxation.

Effects of Exogenous Estrogen and Endogenous Progesterone on the  
Immature Rat's Oviductal Epithelium

In view of the preceeding discussion, it is most likely that the oviducts of the immature rats receiving estradiol treatment for longer than three days were actually being subjected to a biphasic type of hormonal stimulation. During the first three days of estrogen treatment, the oviductal cells were responding to the exogenous estradiol, which was supplemented by a rising output of endogenous estradiol (stimulated by increasing FSH secretion and also possibly be a direct action of the exogenous estradiol on the immature ovarian follicles- Bradbury, 1961; Lane, 1935; Price and Ortiz, 1944).

During the fourth and subsequent days of estradiol treatment however, the oviducts were also being subjected to progestational stimulation. Progesterone appears to have an antagonistic effect on the estrogen-induced changes in the rat oviductal epithelial cells. After two days of progestational influence (i.e. after five days of exogenous estrogen) the cell, nuclear and nucleolar sizes had returned to averages within control values.

Progesterone has been observed to antagonize the actions of estrogen in the oviductal epithelial cells of other species. The height of the oviductal epithelium of ovariectomized rabbits given estradiol plus progesterone for seven days was greater than that of the ovariectomized controls, but was less than that of the rabbits treated with estradiol alone (Gupta, Karkun and Kar, 1969). The same effect was observed in a similar study using ovariectomized cows (McDaniel, Scalzi and Black, 1968).

Besides diminishing the cell growth effects of estradiol, progesterone also appears to antagonize other cell processes which are stimulated by estrogen. Estrogen induces ciliation and hypertrophy of the rhesus monkey fimbrial cells, and this will occur when the ratio of plasma progesterone to plasma estradiol is 10/1. However if the level of progesterone increases until the P/E ratio is 50/1, atrophy and deciliation of the fimbrial cells will occur (Brenner and Resko, 1972).

Progesterone has also been observed to inhibit the estrogen-induced increase in nucleolar size in the luminal epithelial cells of the rat uterus (Tachi, Tachi and Linder, 1972). A similar effect was observed in the present study, and this suggests that progesterone is capable of blocking estrogen-induced increases in protein synthesis in certain cells.

The morphology of the rat oviductal epithelium following six days of estrogen treatment suggested that progesterone (or the combination of estrogenic and progestational stimulation) was inhibiting the release of secretion, and possibly the formation of secretion granules. The cisternae of rough endoplasmic reticulum were markedly dilated with an electron-lucent substance. Several large intracellular microvillous vesicles suggested the coalescence of distended segments of RER. Although secretory granules abound in the oviduct of this rat, they were probably formed under the early estrogenic stimulation, and it will be seen in Part III that the rat oviducts exposed to prolonged progestational stimulation contained many intracellular microvillous vesicles but few granules.

Intracellular mucus inclusions have been described by Spriggs

and Jerrome (1975) in malignant cells found in effusions of the serous cavities. These inclusions are ultrastructurally very similar to those observed in the rat oviduct, and on the basis of histochemistry and ultrastructure, Spriggs and Jerrome interpreted them as the results of inspissation of mucus in vacuoles which were unable to discharge their contents.

Nuclear inclusions were often observed in the oviductal epithelial cells of the immature rats in the present study treated with estradiol for four or more days. Jirsová and Kraus (1974) described similar nuclear inclusions in the oviductal epithelium of mature rats which had been treated for three days with chorionic gonadotropin. This hormone prolongs luteal function; however whether the nuclear inclusions observed in these two studies are a result of progestational stimulation of the cells, can only be a matter for conjecture in the absence of further evidence.

#### Effect of Exogenous Estradiol on the Oviductal Epithelium of the Immature Rabbit

i. Dosage of Estradiol and the Uterine Growth Response. The results of the present investigation into the effects of estrogenic stimulation on the oviduct of the immature rabbit were somewhat confusing. This was primarily because several of the experimental animals showed no increase in uterine weight following hormone treatment. As a uterine growth response provides an indication of estrogenic activity, the absence of such a response in several of the animals made interpretation of the results more difficult.

The daily dosage of 1µg estradiol-benzoate used in the present study was chosen because Noyes, Adams and Walton (1959) found

that this amount of estradiol-benzoate given daily to ovariectomized does in a subcutaneous injection was sufficient to maintain the uterine weight of the animal at the level found in intact estrus does. Their experimental animals weighed on average approximately 3.5kg. Therefore it was decided that 1 $\mu$ g of estradiol given to immature rabbits weighing 1-2kg) would be likely to effect a response in the reproductive tract, and at the same time would be within the physiological levels of estrogen found in the adult animal.

This was not the case, and initially the diverse uterine responses were blamed on the fact that each litter had received estradiol from a different stock solution (i.e. estrogen+oil mixture, see Materials and Methods, p 41). However a recent re-examination of the work by Noyes and his colleagues revealed that they had also experienced much variation in the response to estrogen of the ovariectomized rabbits' uteri. In general, they observed little increase in uterine weight at dosages of less than 0.8 $\mu$ g. At 0.8 $\mu$ g and higher dosages uterine weight increased rapidly, although they found a very wide spread of weights about the means in each treatment group. This extreme variation did not appear to be caused by differences in the length of treatment received. Noyes and his colleagues also found that the uteri of some does failed to show any response to the estrogen injections, even at the high dosage levels.

The experimental rabbits in litter one of the present study showed a step-wise increase in uterine weight following one and two estrogen injections. Only one of the two experimental animals



in litter two showed an increase in uterine weight, whereas none of the four experimental rabbits in litter three showed a response. This does suggest a difference in final concentration of estrogen in the three estrogen-oil stock solutions. But in the light of the evidence presented in the paper by Noyes, Adams and Walton (1959- the report actually deals with estrogen and ova transport in the oviduct), absence of a uterine growth response cannot be taken as conclusive proof of lack of estrogenic stimulation.

ii. Effect of Estradiol Treatment on Cell and Nuclear Volume. All

of the immature rabbits showed increases in cell volume following estrogen treatment, and in only one instance was the difference non-significant. The average nuclear volume of the epithelial cells was also higher in all of the estrogen-treated rabbits, with significant differences observed in six of the eight experimental rabbits.

However significant differences were also observed between the average nuclear volumes of the two control rabbits. There was also no significant decrease in nuclear volume two weeks after ovariectomy. This suggests that nuclear size varies considerably among the rabbit oviductal epithelial cells and cannot be correlated with the level of circulating estrogen.

Oviductal epithelial cell volume however may be a very sensitive indicator of estrogen levels in the immature rabbit. Although there was no change in nuclear size following ovariectomy of an immature animal, there was a dramatic decrease in oviductal epithelial cell volume. This decrease occurred even though the ovaries of this animal contained only primordial follicles and therefore would be secreting only very small amounts of estrogen

(Deanesly, 1972; Eaton and Hilliard, 1971).

Exactly how estrogen affects cell volume in the rabbit oviductal epithelium is not clear. The increase in cell volume following estrogenic stimulation does not appear to be a direct result of an increase in overall protein synthesis (see below). One possibility is that estrogen directly, or indirectly (via general systemic effects) could cause a change in the permeability of the epithelial cell membrane that resulted in different degrees of water imbibition by the cell. Whatever the mechanism involved, it appears that the amount of estrogen necessary to induce a change in cell volume is less than that required to elicit a uterine growth response in the rabbit, or to increase protein synthesis in the oviductal epithelial cells.

iii. Effect of Estradiol Treatment on Protein Synthesis. The experimental animals in litter one appeared to be the only estrogen-treated rabbits in the present study that absorbed enough hormone to raise the level of protein synthesis occurring in the oviductal cells. Twenty-four hours after the first estrogen injection the nucleoli of the epithelial cells had increased in size, and forty-eight hours after hormone treatment had commenced, the RER cisternae were markedly distended with an electron-lucent substance.

The number and size of the secretory granules in the oviductal cells can be taken as an indication of the degree of protein synthesis occurring within these cells. As observed earlier in this report, the nonciliated cells of the immature rabbit's oviduct possess fewer and smaller granules than those of the mature estrus doe. This is probably a reflection of the difference in estrogenic output between the immature and mature rabbit ovaries. Therefore

an increase in protein synthesis in the immature oviductal epithelium understandably follows estrogen administration. What is not completely clear is why this effect was not observed in the oviducts of the other estrogen-treated rabbits. As discussed above, the most likely explanation appears to be a difference in the amount of estrogen received by each animal.

iv. Ovarian Histology and Estradiol Treatment. All of the rabbits used in the present study were the same age at the start of each experiment. However histological examination revealed a considerable variability in ovarian development. This is consistent with other experimental findings. In a study of the postnatal development of the ovary, Deanesly (1972) found that the ovarian follicles and uteri of her 74- and 76-day-old rabbits were smaller than those of the 67-day-old rabbit.

High dosages of estrogen (12,000 Rat Units--see note p3) administered to immature rabbits daily for seven days causes considerable growth and maturation of the primordial follicles present in the ovaries of rabbits at that age (2.5 months--Mazer, Israel and Alpers, 1936). The dosage of estrogen used in the present study was infinitesimal compared to that used by Mazer and his colleagues, and in view of the variability of follicular development seen in the control and experimental rabbits in the present study, there is no justification for any correlation between estrogen treatment and follicular size.

#### Concluding Comment

In the present study the effects of exogenous estradiol on

the oviductal epithelium of the immature rat and rabbit were examined. A study by Verhage et al. (1973b) investigating the effects of estrogen treatment on the epithelium of the immature Beagle's oviduct was the original stimulus for the present experiment. For this reason, this section of the Results and Discussion concludes with a comparison of their results to those presented here.

The oviductal epithelium of the 6- to 8-week-old Beagle pup is composed of morphologically identical cuboidal cells possessing neither cilia nor secretory granules. Administration of estradiol to the pups over a ten day period results in hypertrophy of the cells and the complete differentiation of the epithelium into ciliated and secretory elements (Verhage et al., 1973).

The oviducts of the three-week-old rats and the eight-week-old rabbits used in the present study however were already undergoing differentiation. As far as could be determined from the morphological examination, the estrogen treatment did not alter the process of cytodifferentiation occurring in the oviducts of these species. Estrogen treatment did however supplement the levels of the hormone being produced by the animals' own immature ovaries, and subsequently amplified the process of protein synthesis occurring in the oviductal cells.

None of the immature rats, rabbits and pups used in these experiments had been ovariectomized. This fact introduced an additional variable into the experiments, which critically affected the results in the present study using immature rats. Evidence presented in this discussion strongly suggested that the exogenous estrogen had provoked increasing gonadotropin release, which subsequently stimulated the immature rats' ovaries to

secrete progesterone. This effect was not observed in the rabbit, for a neural stimulus provoked by coitus is necessary for the stimulation of LH secretion in this species, and ovulation can therefore not be induced by estrogen injections (Everett, 1961).

One of the effects of the combination of estrogenic and progestational stimulation of the rat oviduct was a lowering of the height of the epithelium. In the Beagle pup study, nonciliated cell and nuclear height peaked after three days of estrogen treatment, and steadily declined thereafter. The differentiation of ciliated cells was delayed in comparison to that of the secretory cells, and hypertrophy of the ciliated cells did not reach a maximum until after six days of estrogen treatment. Again however, after this time the cell and nuclear volumes of the ciliated cells steadily decreased.

Verhage and his colleagues did not comment on this; nor did they make any reference to the effects of exogenous estrogen on the ovaries of the immature Beagles. They did say however that the epithelial cells of the oviducts of the 6- to 8-week-old Beagles were 'mature' in the sense that they responded to estrogen treatment in the same manner that the epithelium of the mature bitch responds to rising levels of plasma estrogen during proestrus.

The oviduct of the mature Beagle bitch undergoes a complete differentiation during the proestrus stage of each estrus cycle, and following ovulation, dedifferentiates during metestrus (Verhage et al., 1973a). If it is assumed that the estradiol treatment affected the hypothalamico-pituitary-ovarian axis of the immature

Beagle in much the same way as it affected the immature rat, then the decline in epithelial height observed in their study could be attributed to ovarian progesterone release. This is supported by the fact that Verhage and his colleagues (Ibid.) have already found a strong correlation between the regression and atrophy of the oviductal epithelium observed during metestrus, and the rising levels of plasma progesterone.



FIGURES 26-42

Figure 26: Effect of estrogen treatment on cell volume in the oviductal epithelium of the immature rat. (Estrogen treatment: subcutaneous injection of 1µg estradiol benzoate in 0.1ml oil/day.)

# EFFECT OF ESTROGEN TREATMENT ON CELL VOLUME

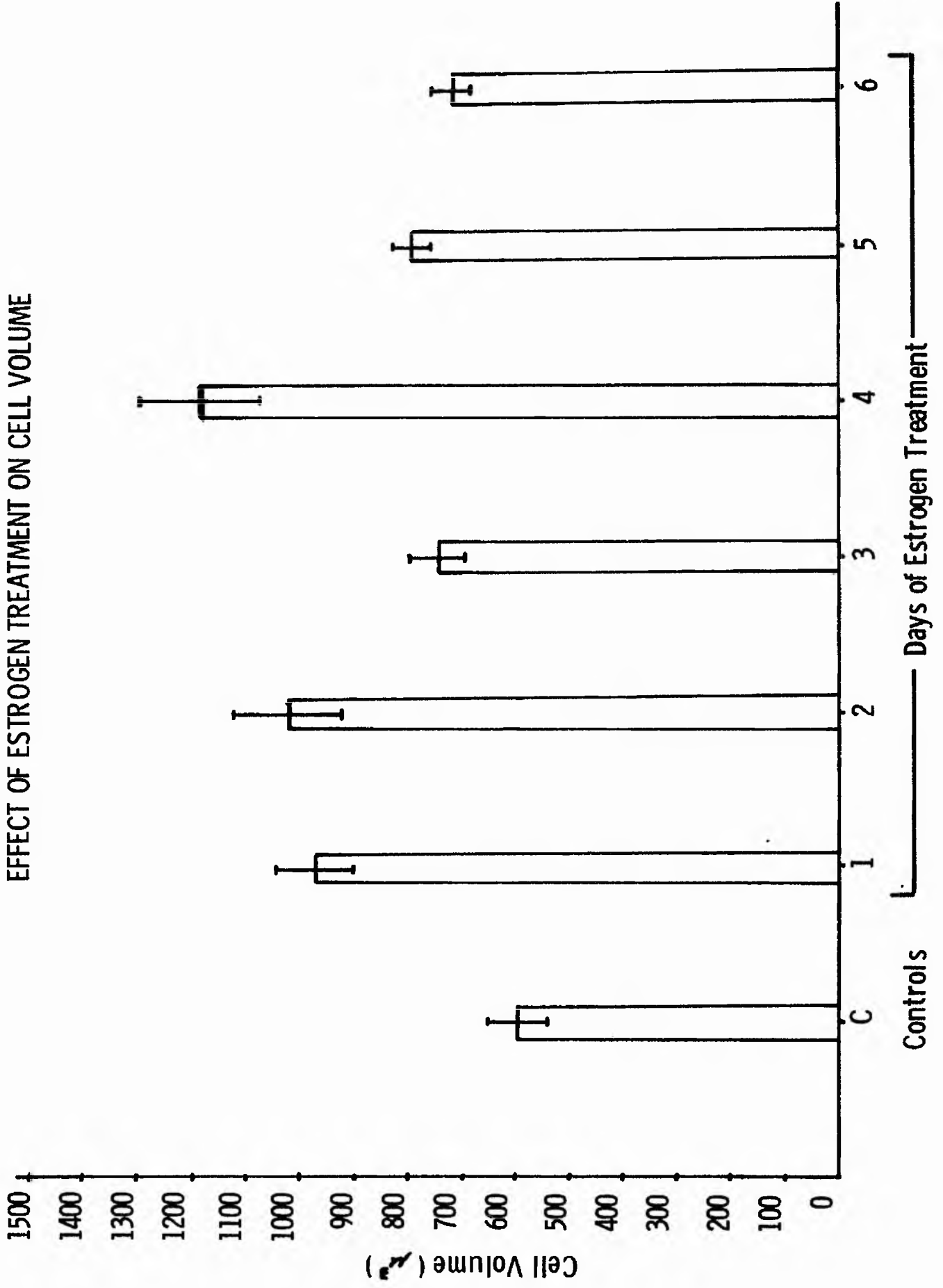


Figure 27: Effect of estrogen treatment on nuclear volume in the oviductal epithelium of the immature rat. (Estrogen treatment: subcutaneous injection of 1µg estradiol benzoate in 0.1ml oil/day.)

# EFFECT OF ESTROGEN TREATMENT ON NUCLEAR VOLUME

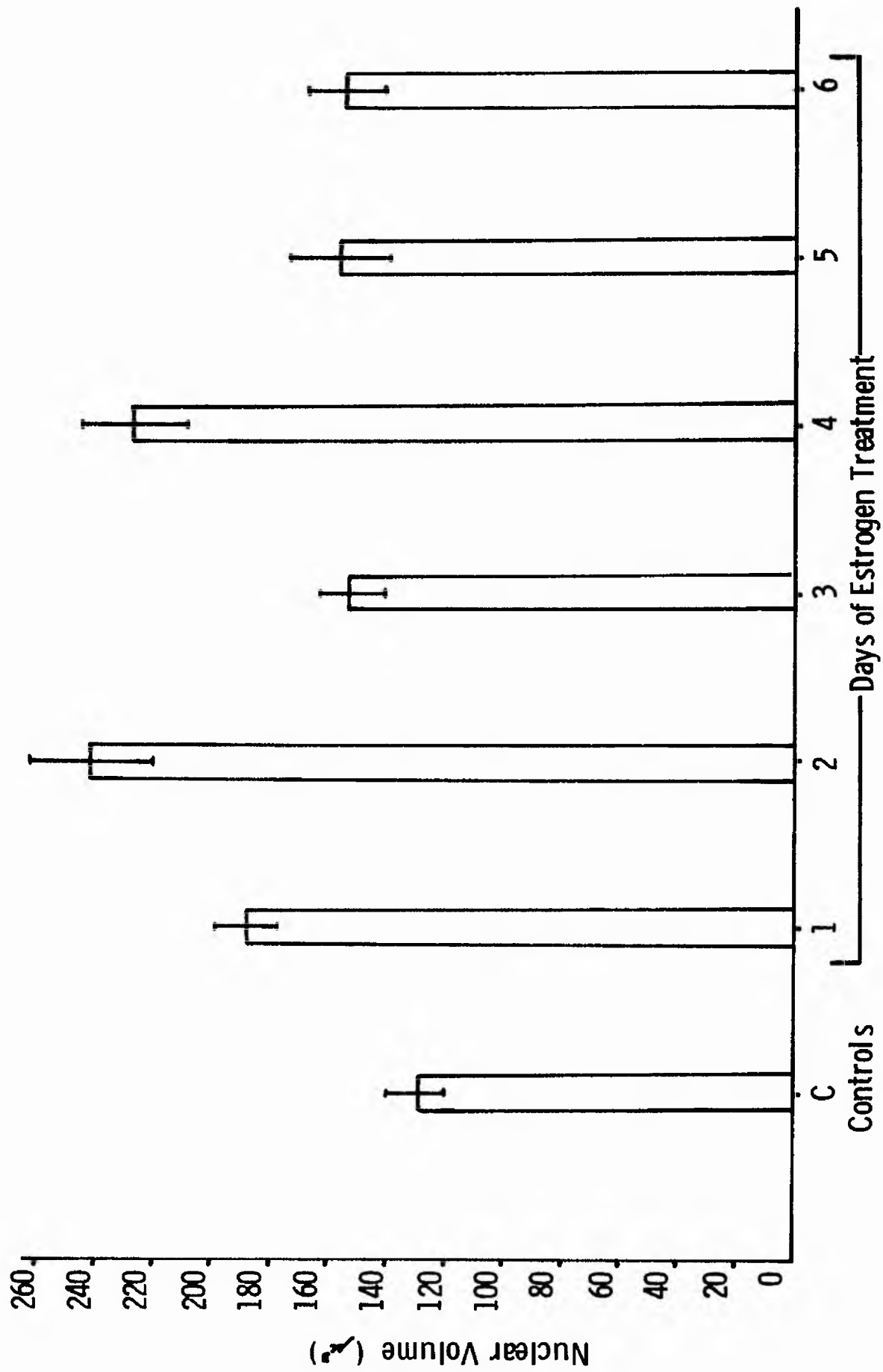


Figure 28: Effect of estrogen treatment on nucleolar diameter in the oviductal epithelium of the immature rat. (Estrogen treatment: subcutaneous injection of 1 $\mu$ g estradiol benzoate in 0.1ml oil/day.)



# EFFECT OF ESTROGEN TREATMENT ON NUCLEOLAR DIAMETER

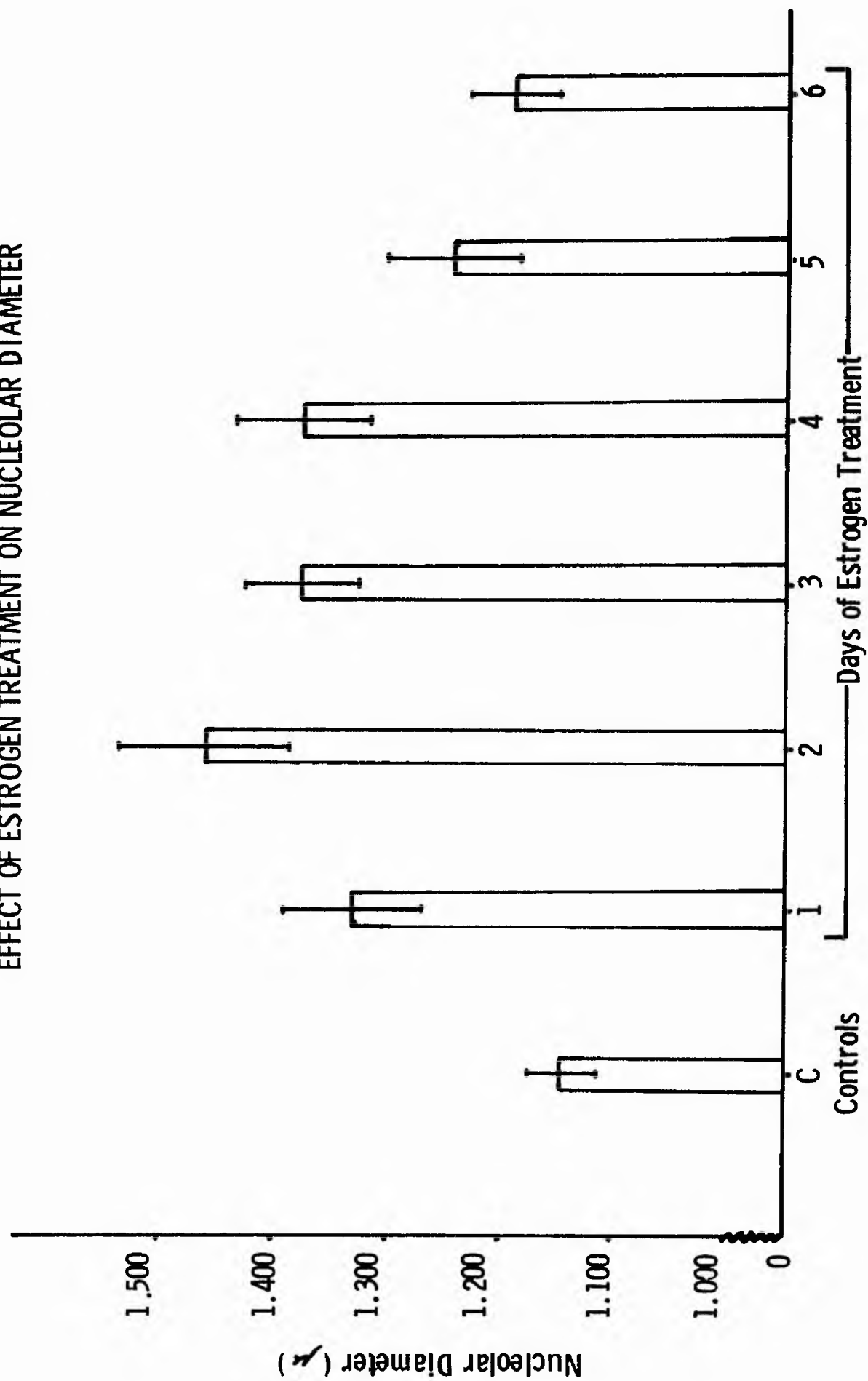


Figure 29: Effect of estrogen treatment on the weight of the uterus of the immature rat. (Estrogen treatment: subcutaneous injection of 1 $\mu$ g estradiol benzoate in 0.1ml oil/day.)

# EFFECT OF ESTROGEN TREATMENT ON UTERINE WEIGHT

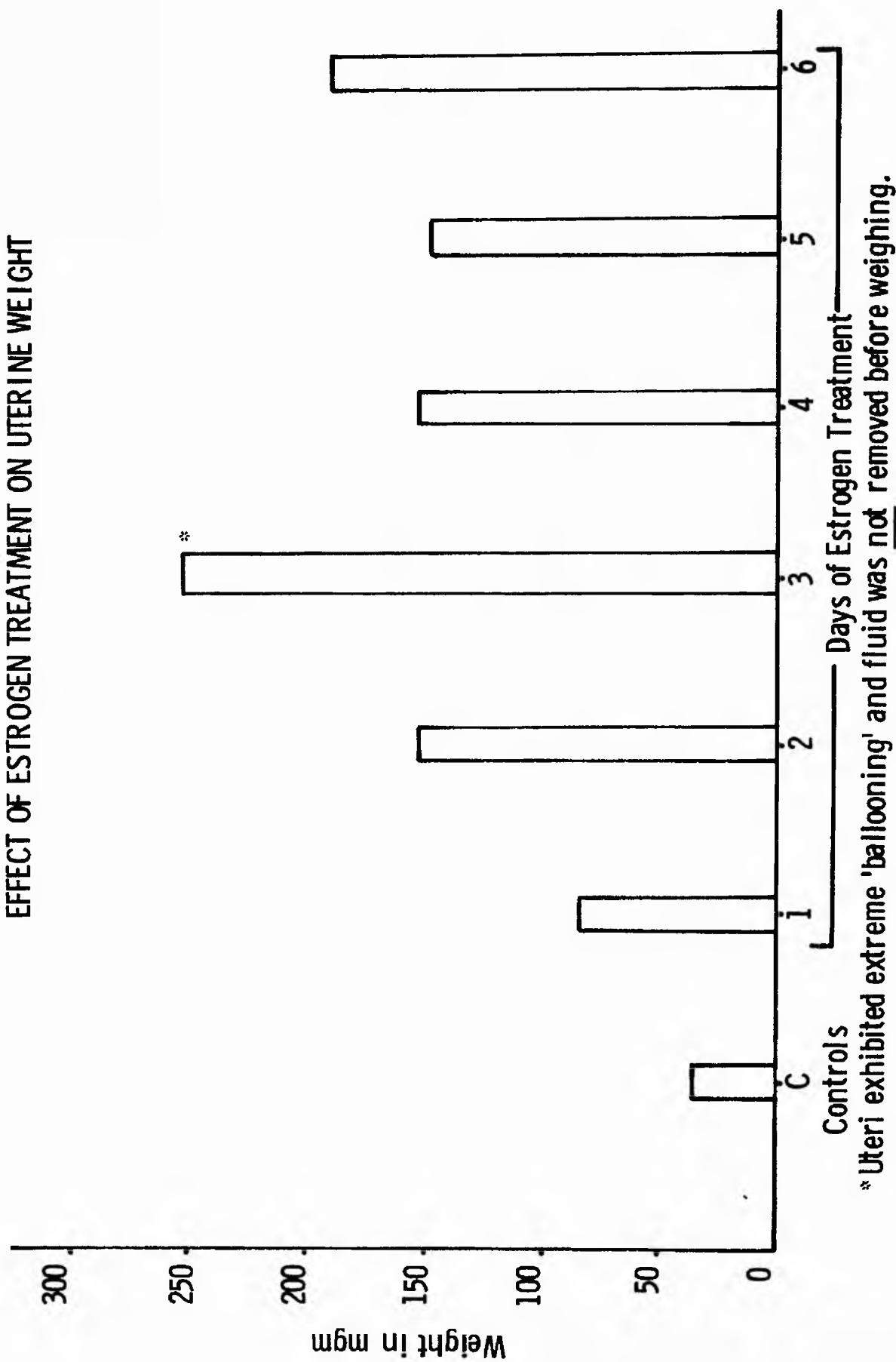


Figure 30: Oviductal epithelium of an immature rat. Oneday oil

only. UA&LC. X6510

SG- secretory granules

→ - small mucus granules



SG

30

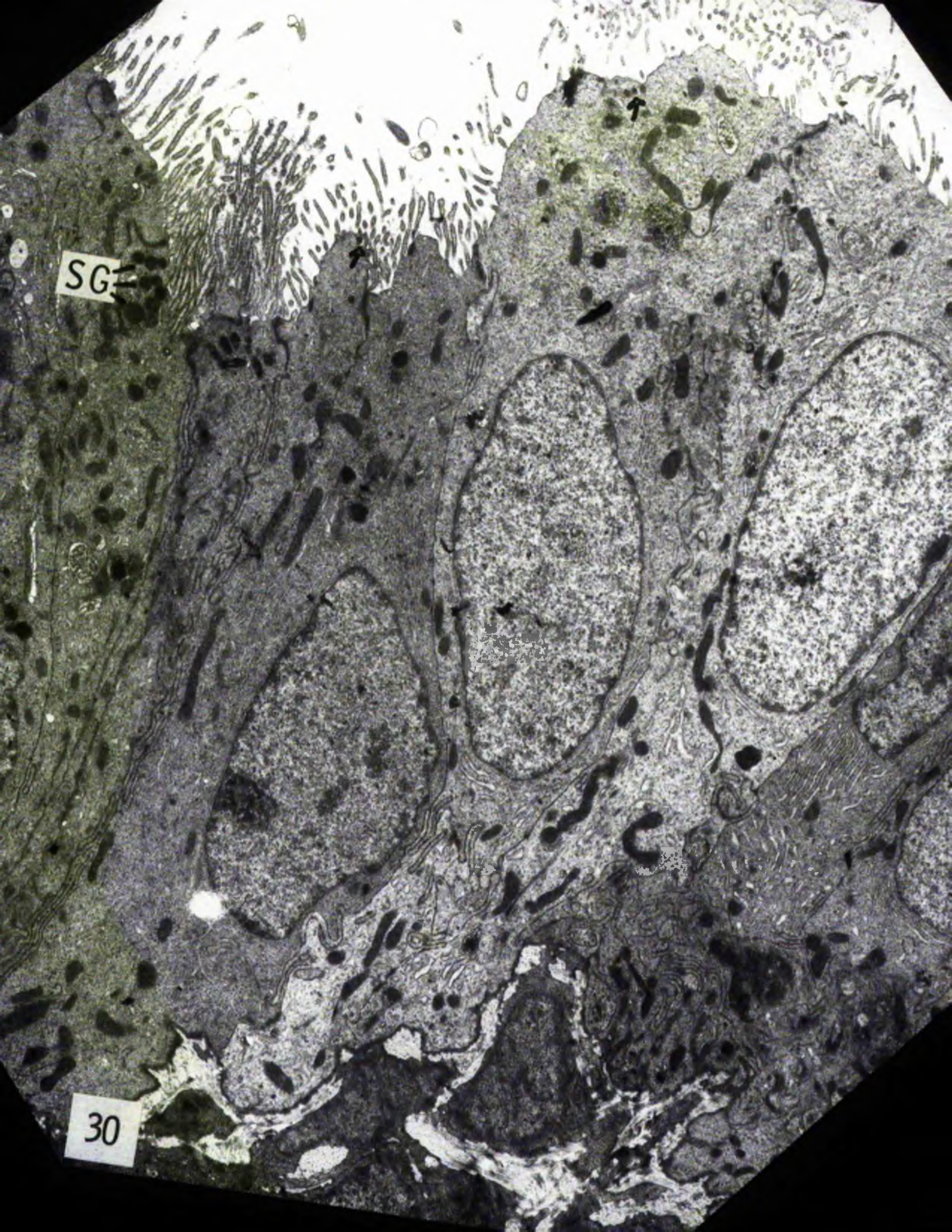




Figure 31: Oviductal epithelium of an immature rat. Two days estrogen. UA&LC. X6510

M- mitosis

rer- rough endoplasmic reticulum

→ - small mucus granules



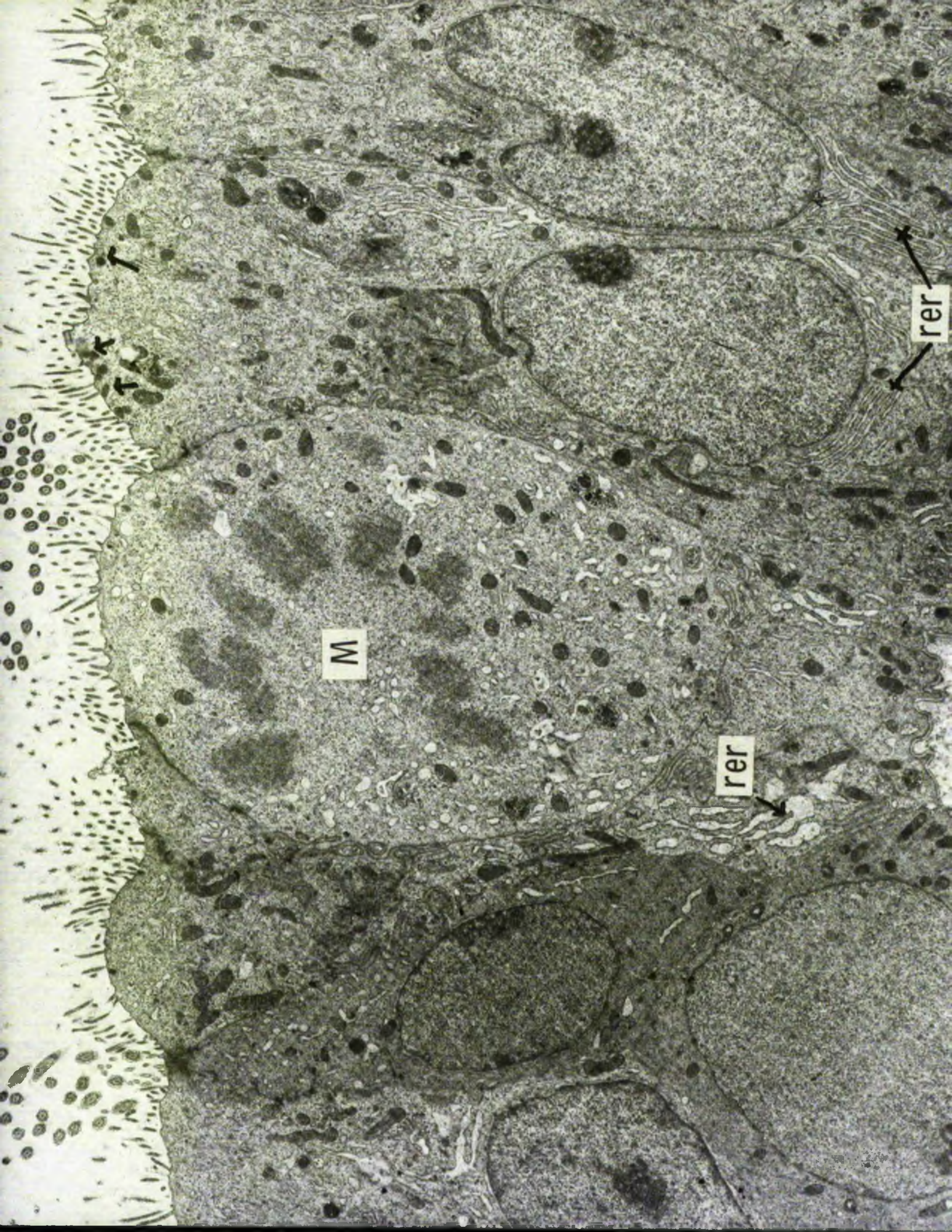




Figure 32: Oviductal epithelium of an immature rat. Five days

estrogen. UA&LC. X6510

CV- ciliary vacuole

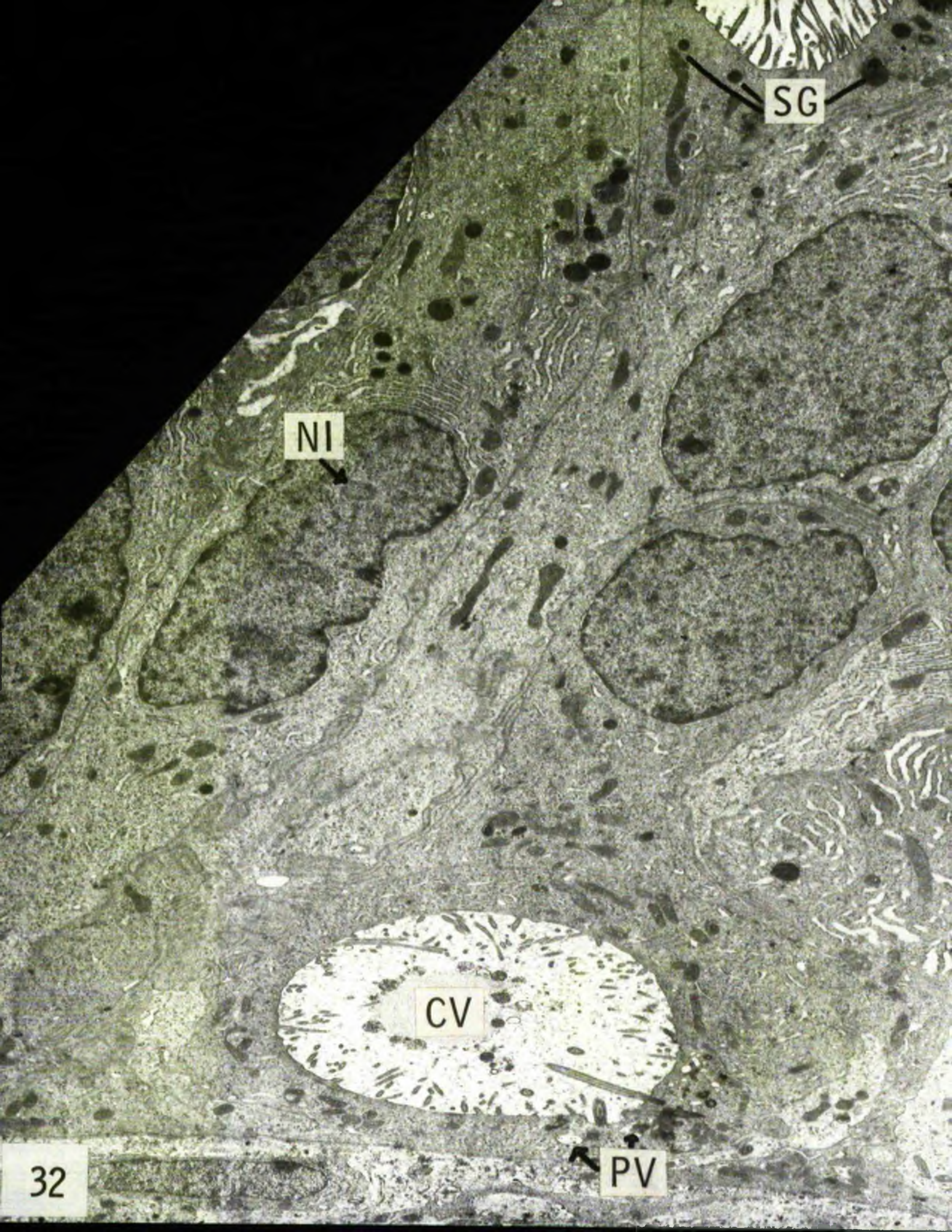
NI- nuclear inclusion

PV- primary ciliary vesicle

rer- rough endoplasmic reticulum

SG- secretory granules





NI

SG

CV

PV



Figure 33: Oviductal epithelium of an immature rat. Six days estrogen. UA&LC. X3710

MV- microvillous vesicle

NI- nuclear inclusion

rer- rough endoplasmic reticulum

SG- secretory granules



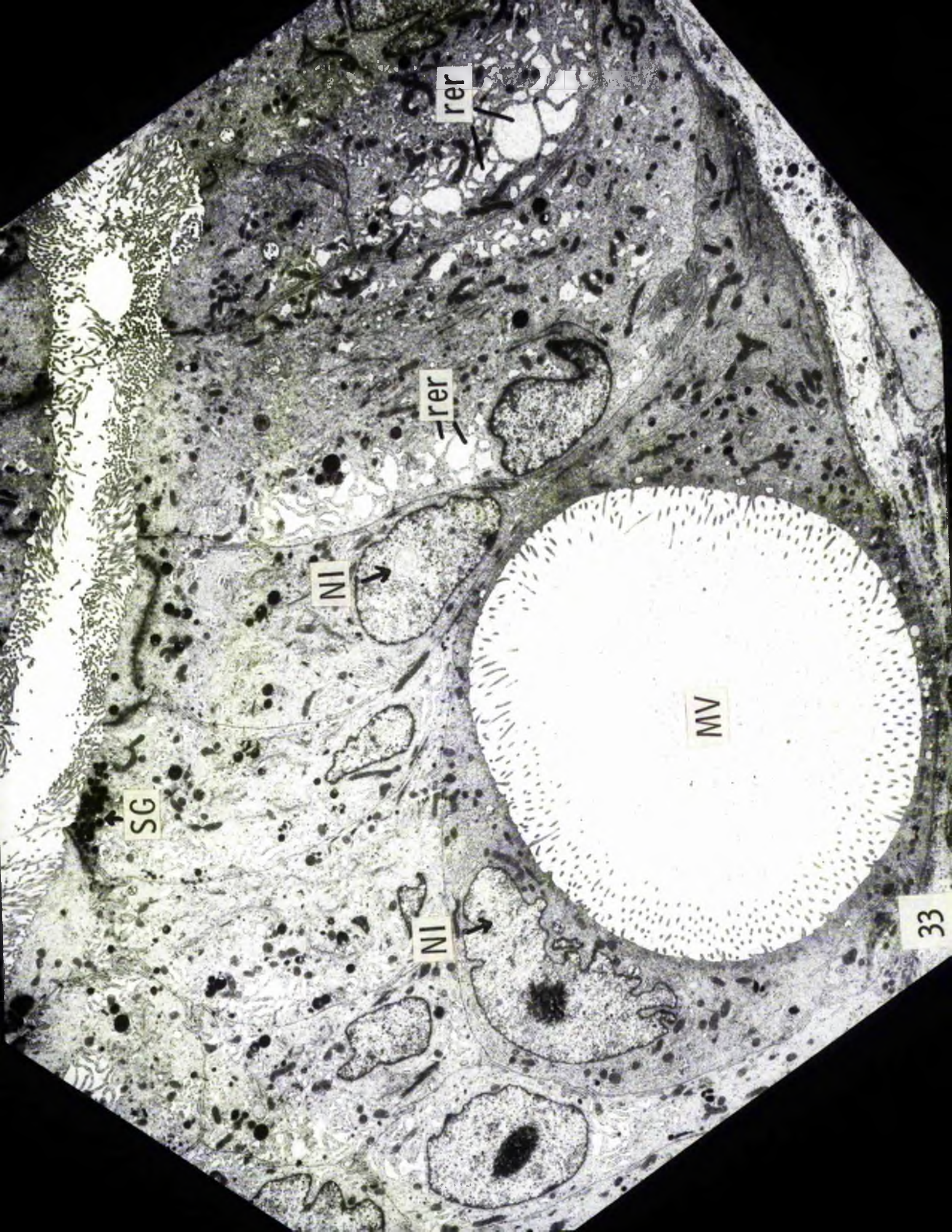




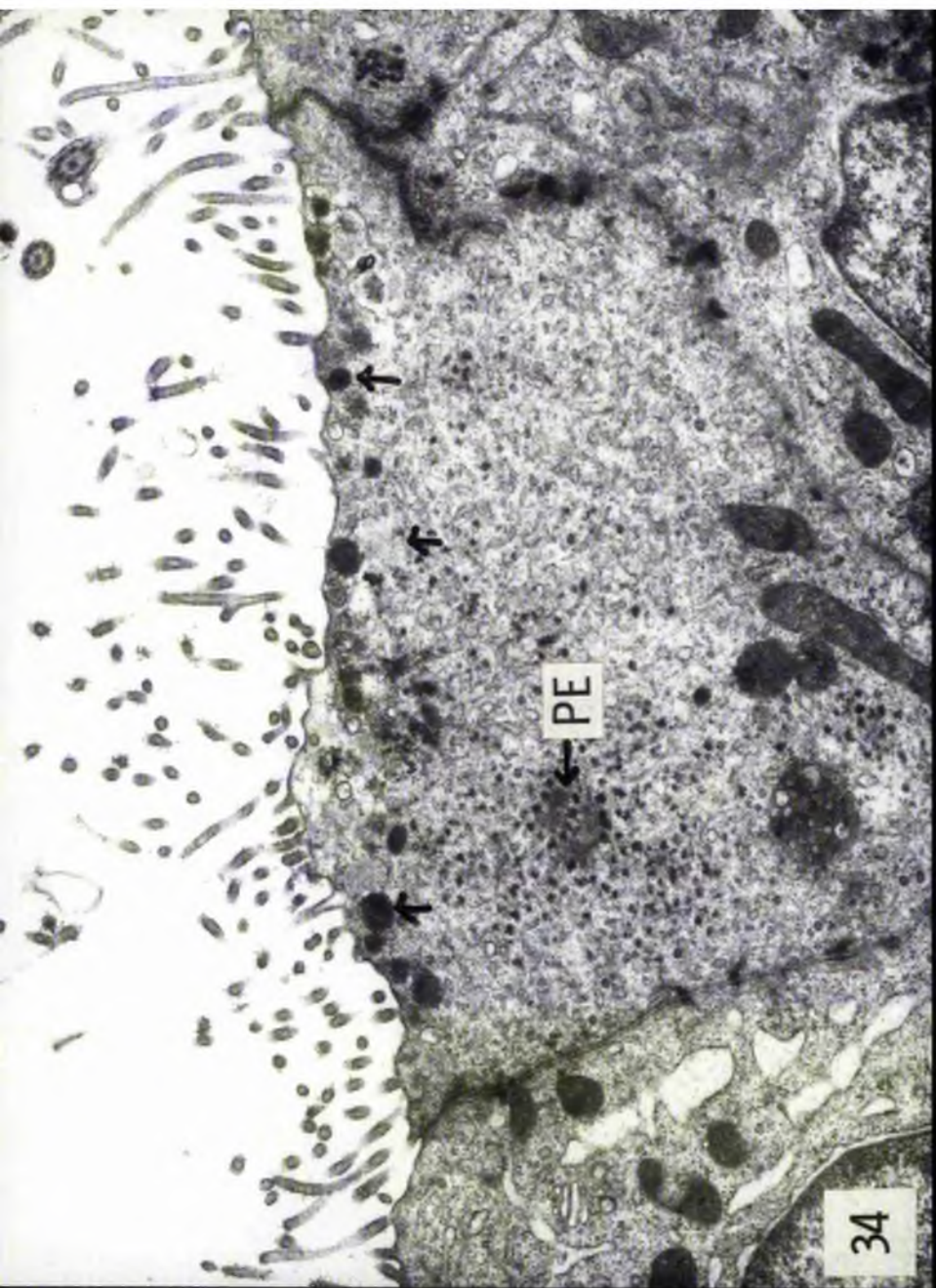
Figure 34: Ciliogenic cell in the oviductal epithelium of an  
immature rat. UA&LC. X27,615

PE- proliferative elements

→ - small mucus granules

Figure 35: Nuclear inclusions (NI) in the oviductal epithelium  
of an immature rat. Four days estrogen. UA&LC X27,615





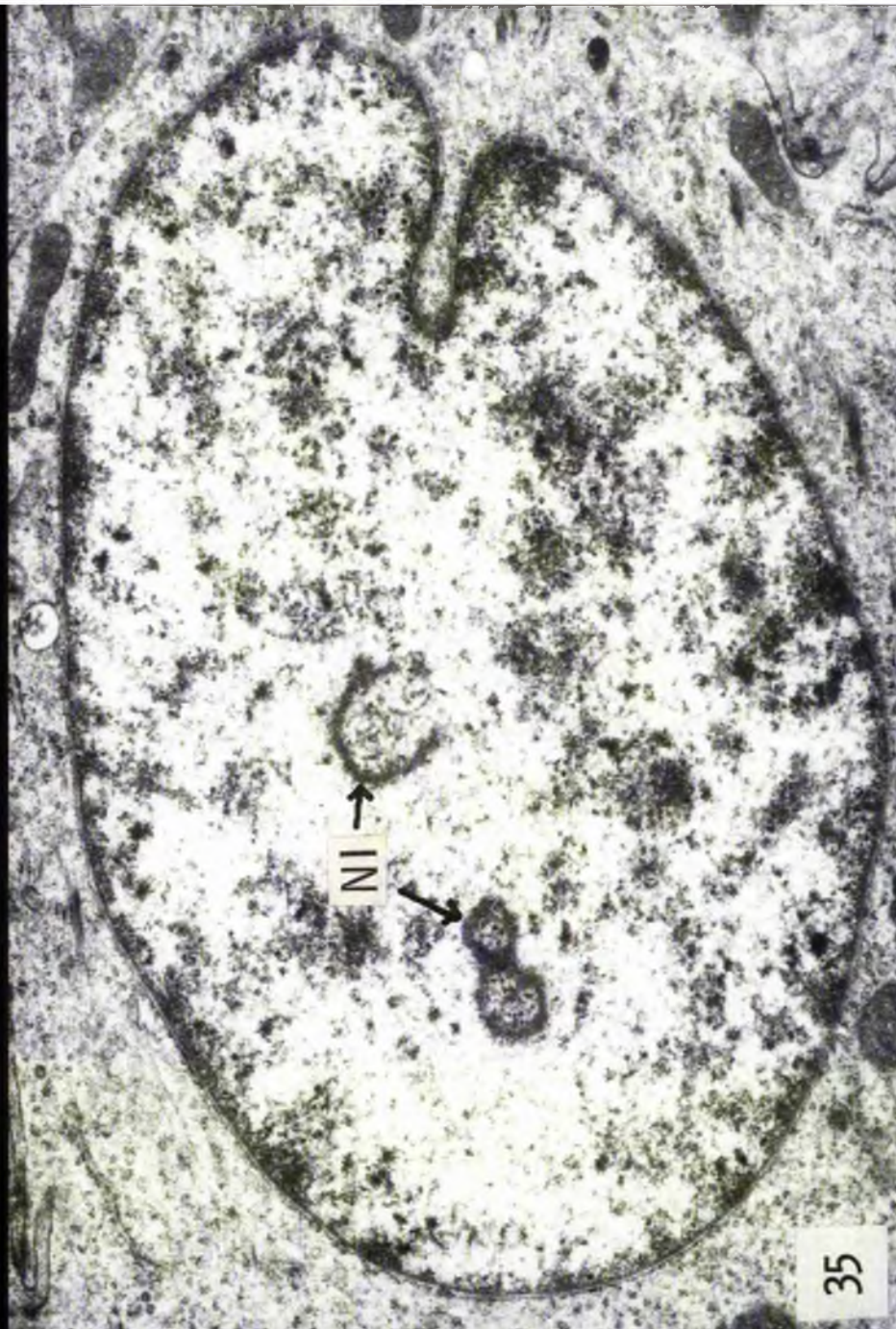
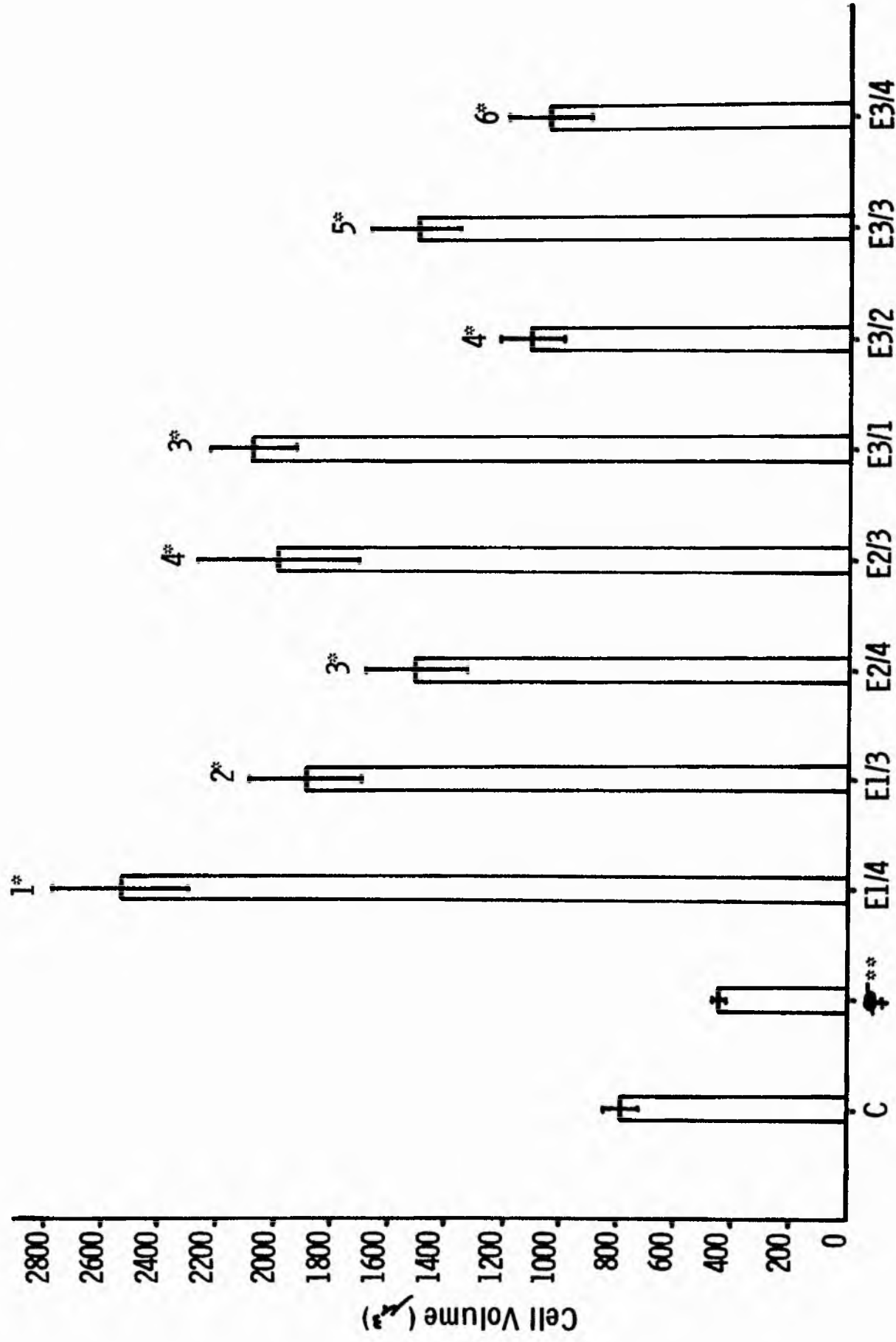


Figure 36: Effect of estrogen treatment on cell volume in the oviductal epithelium of the immature rabbit. (Estrogen treatment: subcutaneous injection of 1 $\mu$ g estradiol benzoate in 0.1ml oil/day.)

# EFFECT OF ESTROGEN TREATMENT ON CELL VOLUME

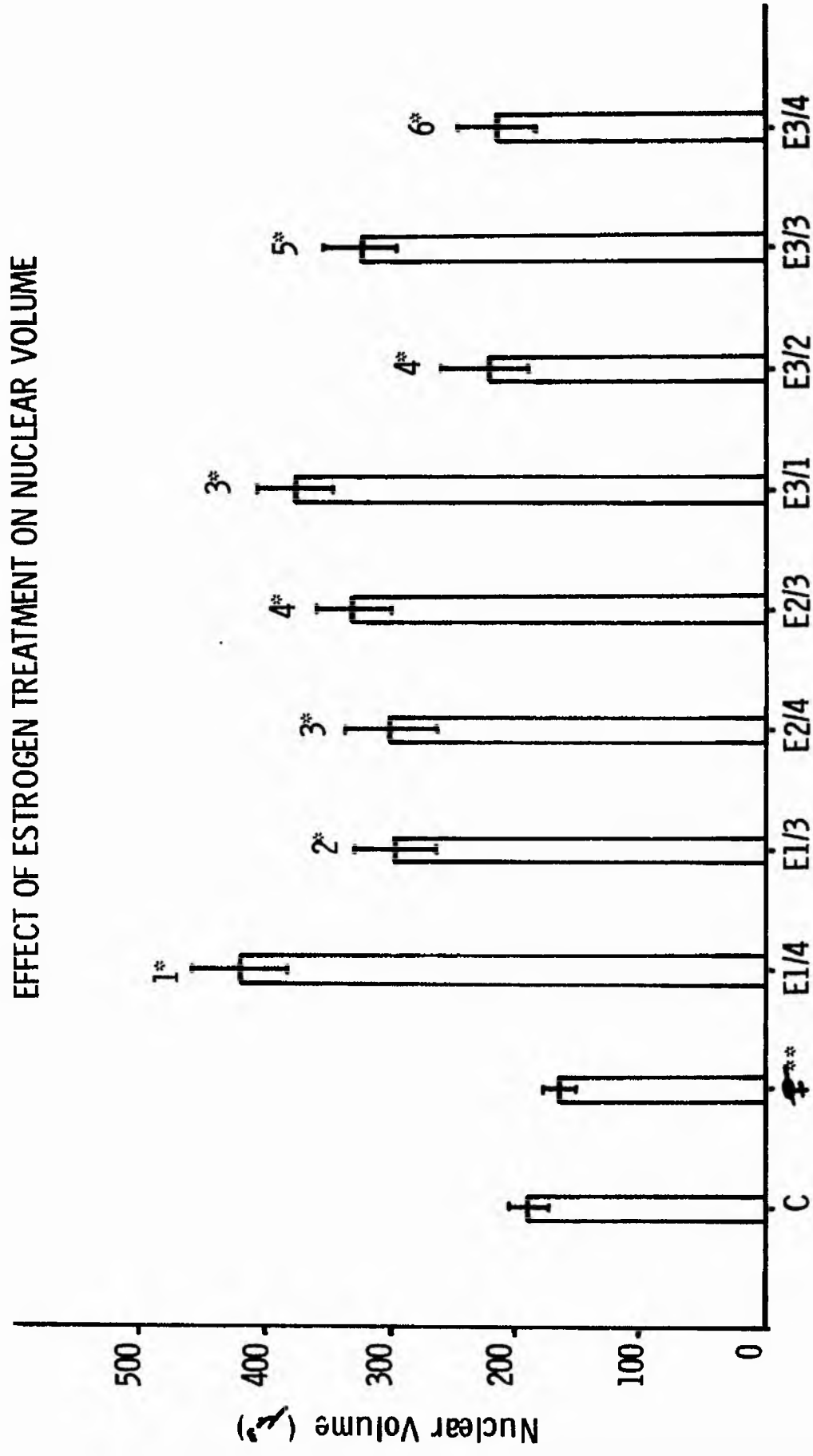


C- controls    \* - days of estrogen treatment    \*\* - sacrificed two weeks after bilateral ovariectomy



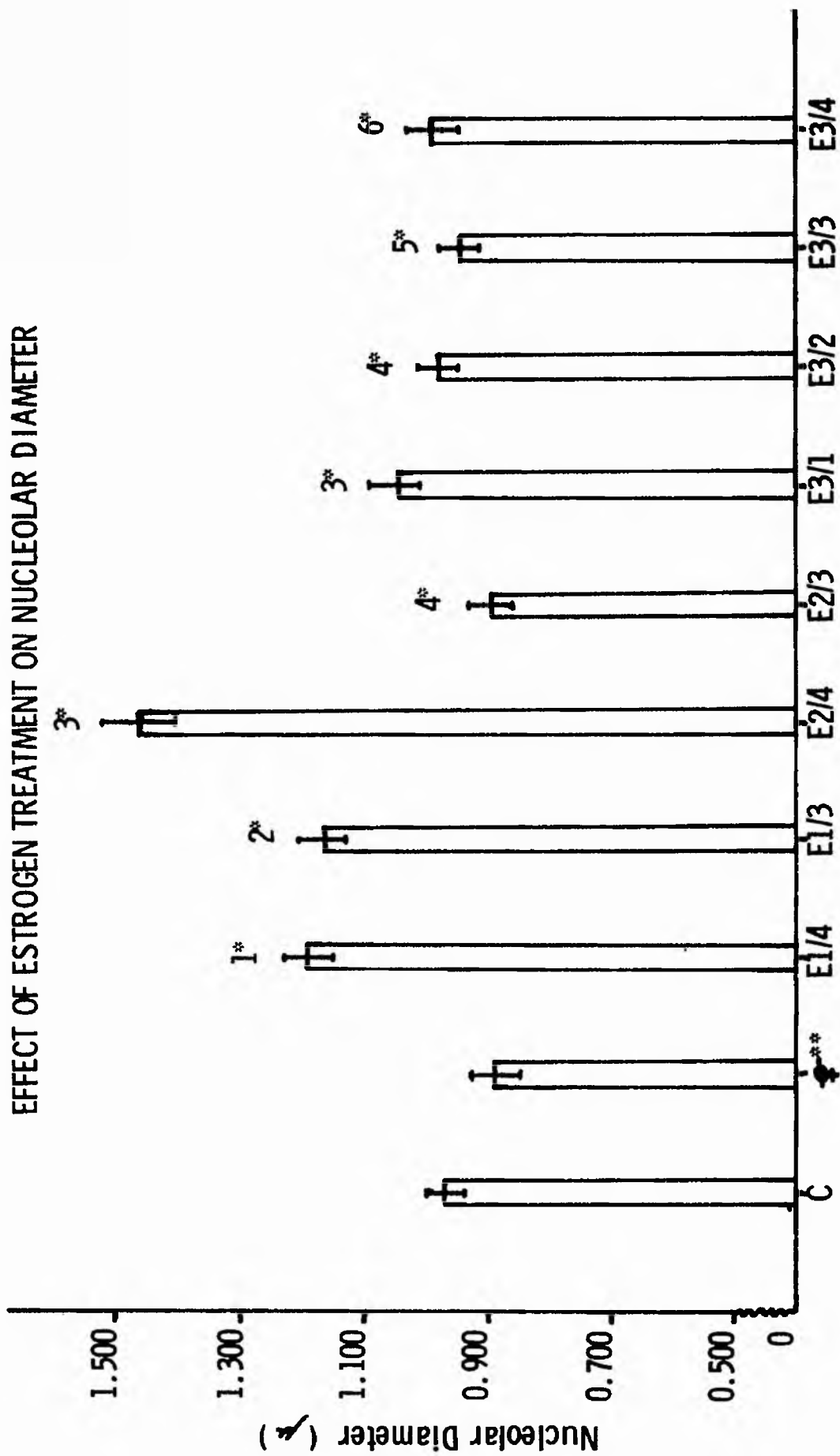
Figure 37: Effect of estrogen treatment on nuclear volume in the oviductal epithelium of the immature rabbit. (Estrogen treatment: subcutaneous injection of 1µg estradiol benzoate in 0.1ml oil/day.)





C- controls      \* -days of estrogen treatment  
 \*\* - sacrificed two weeks after bilateral ovariectomy

Figure 38: Effect of estrogen treatment on nucleolar diameter in the oviductal epithelium of the immature rabbit. (Estrogen treatment: subcutaneous injection of 1µg estradiol benzoate in 0.1ml oil/day.)



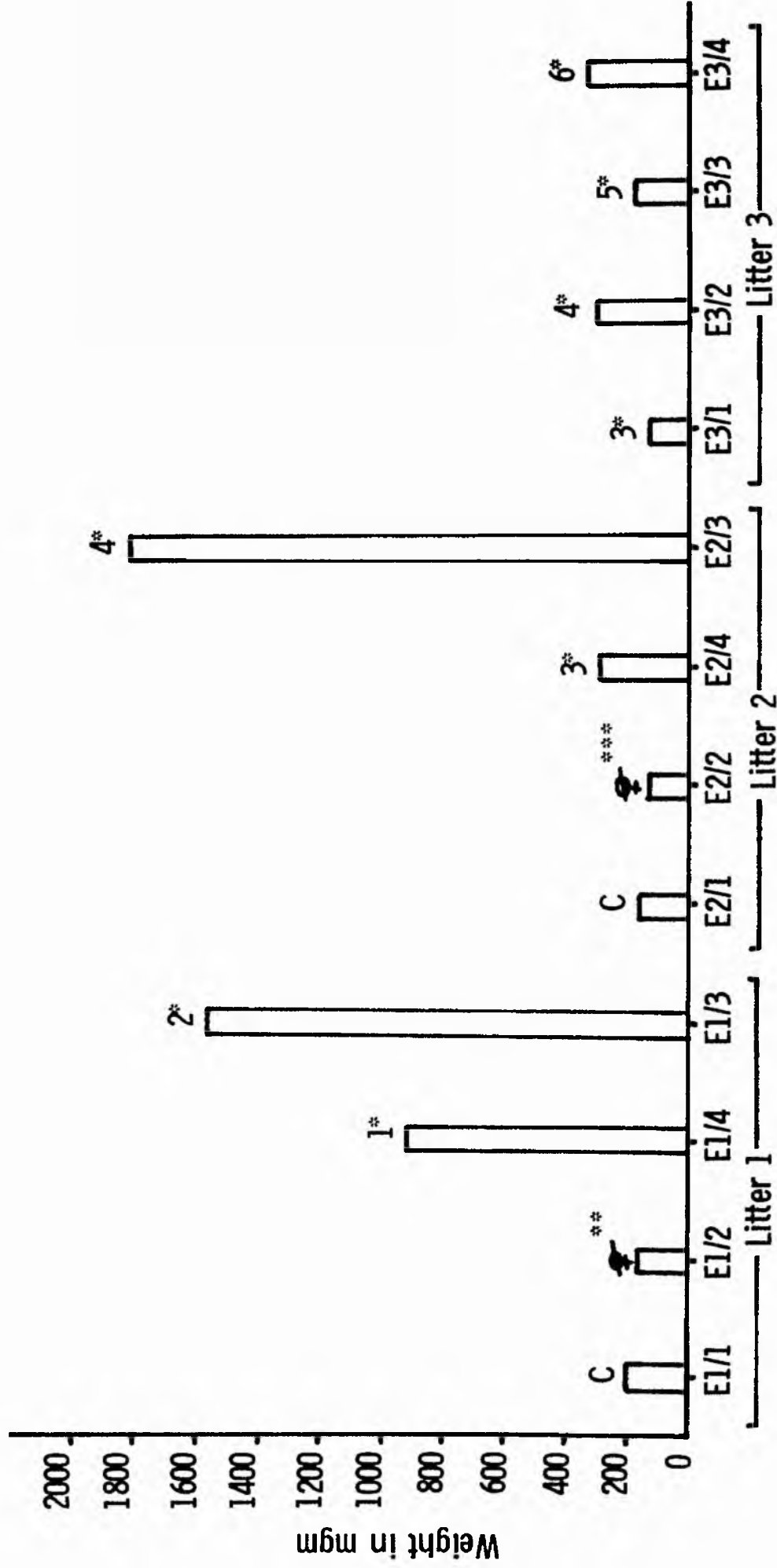
C-controls

\* - days of estrogen treatment

\*\* - sacrificed two weeks after bilateral ovariectomy

Figure 39: Effect of estrogen treatment on the weight of the uterus of the immature rabbit. (Estrogen treatment: subcutaneous injection of 1 $\mu$ g estradiol benzoate in 0.1ml oil/day.)

# EFFECT OF ESTROGEN TREATMENT ON UTERINE WEIGHT



C - control    \* - days of estrogen treatment    \*\* - sacrificed one week after bilateral ovariectomy  
 \*\*\* - sacrificed two weeks after bilateral ovariectomy



Figure 40: Oviductal epithelium (ampulla) two weeks after bilateral ovariectomy of an immature rabbit. UA&LC. X6510



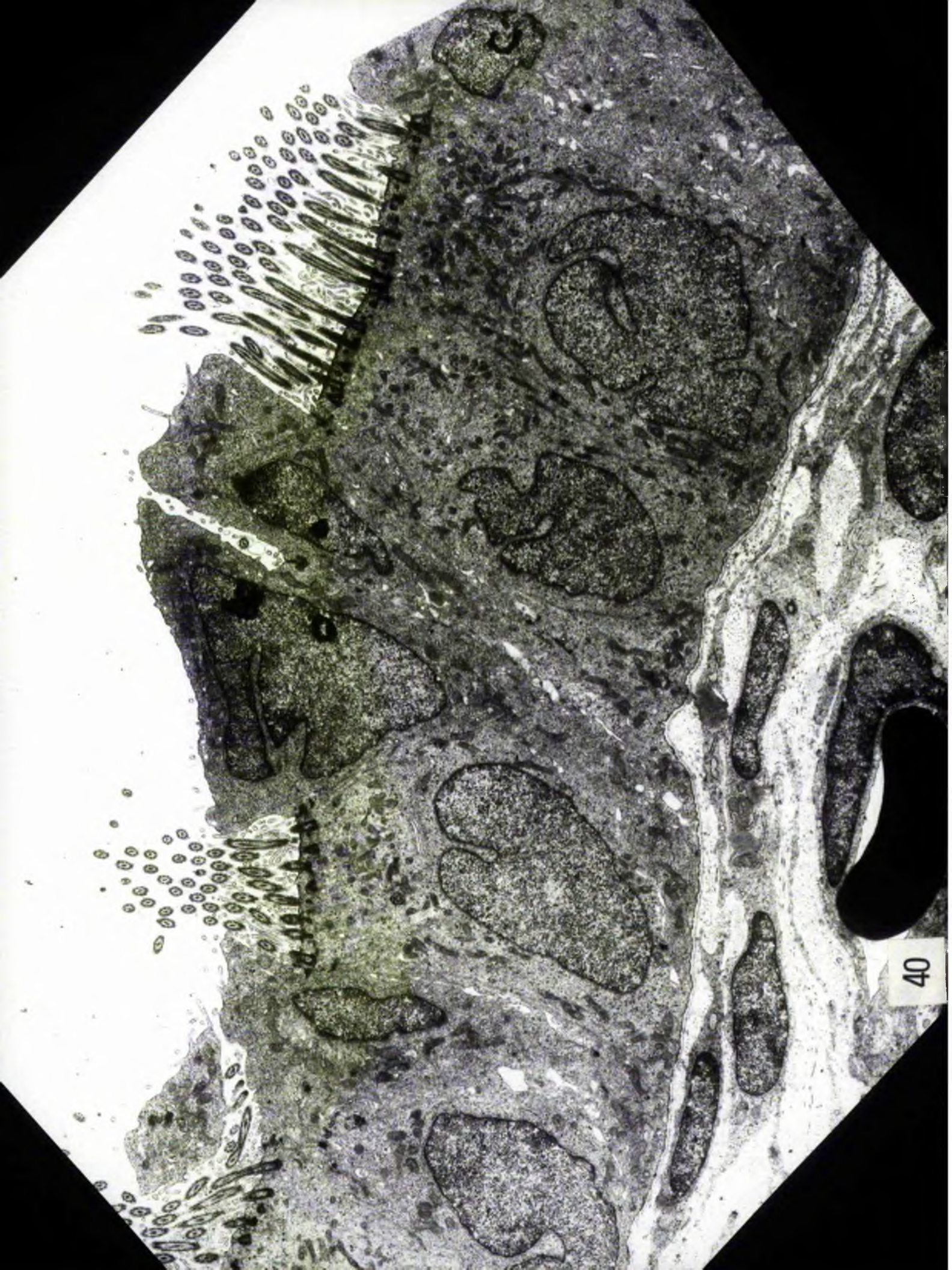
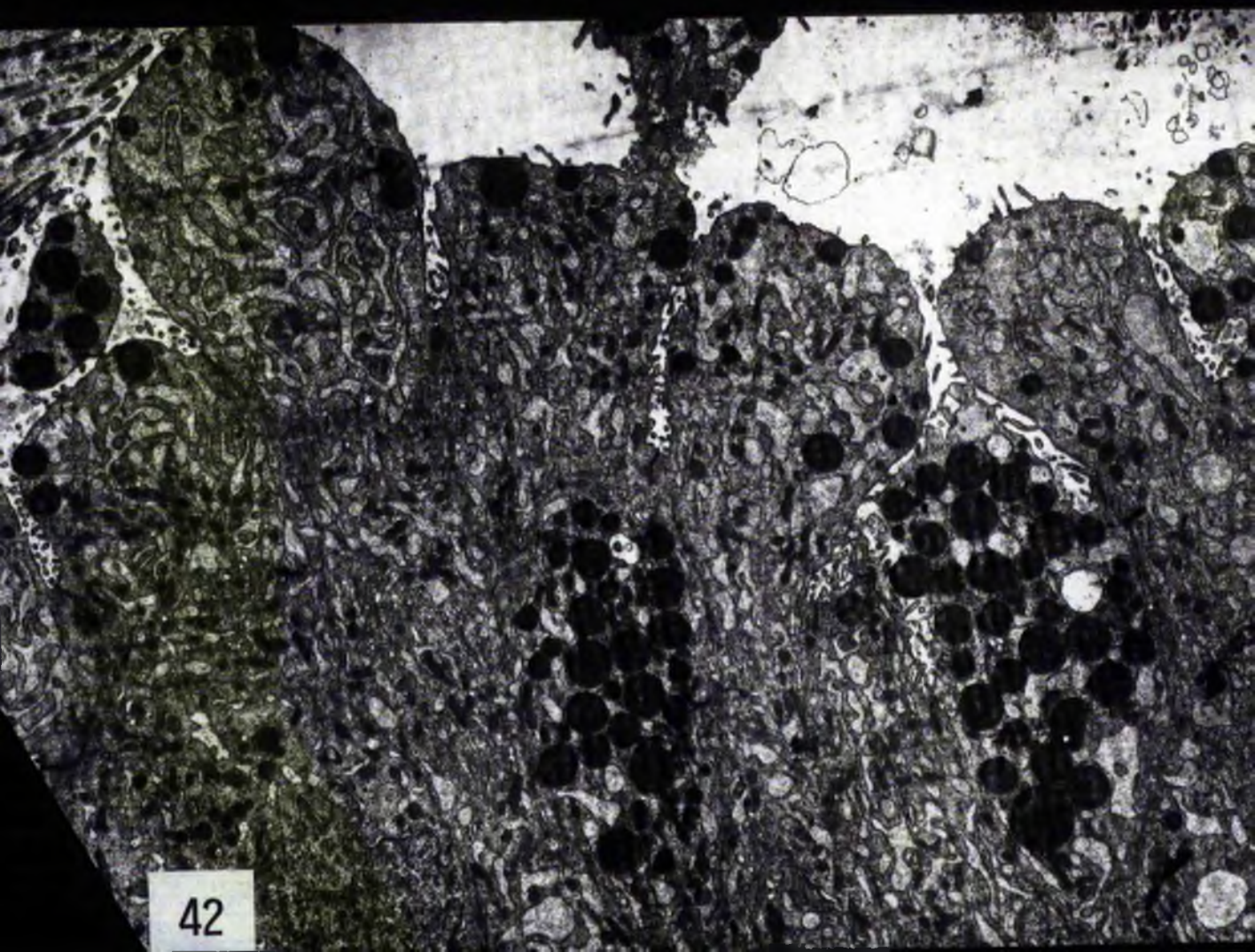




Figure 41: Nucleus and nucleolus from the oviductal epithelium  
of rabbit E2/4 demonstrating improper fixation (see text,  
p. 89). UA&LC. X31,560

Figure 42: Oviductal epithelium of an immature rabbit. Two  
days estrogen. UA&LC. X5,580



### III. THE EFFECTS OF LONG-TERM ESTROGEN ADMINISTRATION ON THE EPITHELIUM OF THE RAT AND RABBIT OVIDUCTS

#### Effect of Subcutaneous Implantation of Pellets of Estradiol on the Body Weight and on the Histology of the Pituitary, Ovaries, Uterus and Mammary Glands of the Rat

i. Body Weight. Although they were not weighed at the time of estradiol implantation, all of the rats used in the second long-term experiment (see Materials and Methods) weighed between 200-250gm on arrival in the animal house. As they were randomly assigned to either experimental or control groups, it can be assumed that at the start of the experiment there were no significant differences in body weight between the two groups.

Following one month of estrogen treatment however, the control rats weighed significantly ( $p < 0.001$  by t-test) more than the experimental rats (Table IV, Fig. 43). The control rats remained significantly heavier than the estrogen-treated rats throughout the four month period. However the effects of the estrogen treatment on body weight appeared to be greatest during the first month, for thereafter there was only a slight increase in the difference between the average weights (Fig. 43).

ii. Pituitary Weight and Histology. The average weight of the pituitaries of the control animals showed only slight variation during the four months of the second long-term experiment. In contrast, the pituitaries of the estrogen-treated rats steadily increased in weight, and at all times were significantly ( $P < 0.05$  by t-test) heavier than the glands of the control animals (Table IV,



Table IV: Effect of Estrogen Treatment on Body Weight, Pituitary Weight, Combined Weight of Both Ovaries, Combined Weight of Both Oviducts and Weight of Uterus

TREATMENT		MEAN WEIGHT (gms) $\pm$ S.E.
(Figures in parentheses indicate sample number)		
Experimental- One Month Estrogen		
Body Weight	(10)	244.600 $\pm$ 4.507 <del>44</del>
Pituitary	(8)	0.019 $\pm$ 0.002**
Ovaries	(10)	0.073 $\pm$ 0.008 <del>44</del>
Oviducts	(10)	0.033 $\pm$ 0.003
Uterus	(10)	0.655 $\pm$ 0.044**
Controls		
Body Weight	(6)	290.667 $\pm$ 8.040
Pituitary	(6)	0.011 $\pm$ 0.000
Ovaries	(6)	0.104 $\pm$ 0.008
Oviducts	(6)	0.029 $\pm$ 0.001
Uterus	(6)	0.397 $\pm$ 0.035
Experimental- Two Months Estrogen		
Body Weight	(10)	242.700 $\pm$ 3.944 <del>44</del>
Pituitary	(10)	0.023 $\pm$ 0.003*
Ovaries	(10)	0.019 $\pm$ 0.003 <del>44</del>
Oviducts	(10)	0.030 $\pm$ 0.001
Uterus	(9)	0.713 $\pm$ 0.025**
Controls		
Body Weight	(6)	314.333 $\pm$ 7.074
Pituitary	(5)	0.012 $\pm$ 0.001
Ovaries	(5)	0.107 $\pm$ 0.008
Oviducts	(5)	0.030 $\pm$ 0.001
Uterus	(5)	0.513 $\pm$ 0.054
Experimental- Three Months Estrogen		
Body Weight	(10)	254.100 $\pm$ 4.545 <del>44</del>
Pituitary	(10)	0.028 $\pm$ 0.002***
Ovaries	(10)	0.018 $\pm$ 0.002 <del>44</del>
Oviducts	(9)	0.029 $\pm$ 0.001
Uterus	(8)	0.667 $\pm$ 0.038*
Controls		
Body Weight	(6)	316.000 $\pm$ 10.780
Pituitary	(6)	0.012 $\pm$ 0.001
Ovaries	(6)	0.085 $\pm$ 0.002
Oviducts	(6)	0.034 $\pm$ 0.002
Uterus	(6)	0.491 $\pm$ 0.053

Table IV (Continued)

TREATMENT		MEAN WEIGHT (gms) $\pm$ S.E.
Experimental- Four Months Estrogen		
Body Weight	(9)	250.889 $\pm$ 10.481 <del>//</del>
Pituitary	(9)	0.031 $\pm$ 0.006*
Ovaries	(8)	0.012 $\pm$ 0.001 <del>//</del>
Oviducts	(9)	0.031 $\pm$ 0.003
Uterus	(8)	0.773 $\pm$ 0.058*
Controls		
Body Weight	(6)	334.167 $\pm$ 9.610
Pituitary	(6)	0.011 $\pm$ 0.001
Ovaries	(6)	0.095 $\pm$ 0.011
Oviducts	(6)	0.033 $\pm$ 0.003
Uterus	(6)	0.593 $\pm$ 0.023

\* Significantly higher than controls,  $p < 0.05$  by t-test.

\*\* Significantly higher than controls,  $p < 0.01$  by t-test.

\*\*\* Significantly higher than controls,  $p < 0.001$  by t-test.

~~/~~ Significantly lower than controls,  $p < 0.05$  by t-test.

~~//~~ Significantly lower than controls,  $p < 0.001$  by t-test.

Fig. 43).

Following one month of estrogen treatment there was a decline in the number of PAS +ve cells observed in the anterior pituitary of the rat. Those present were mainly the red PAS +ve beta cells. (Note: The Iron PAS-Orange G method used in the present study stains alpha-cell (i.e. somatotropes and luteotropes or prolactin cells) granules orange, beta-cell (i.e. thyrotropes) granules PAS +ve red, and delta cell (i.e. gonadotropes) granules PAS +ve purple- see Purves, 1966.) There was also an apparent rise in the number of alpha cells. In the control rats alpha cells were found to be concentrated mainly in the periphery of the gland. Following one month of estrogen treatment, alpha cells were observed throughout the anterior pituitary.

Beta and delta cells were exceedingly rare in the pituitaries of rats treated with estrogen for two months. Alpha cell numbers were still elevated in the glands of these rats, but had apparently decreased in the pituitaries of rats treated for three months with the hormone.

Following three and four months of estrogen treatment the anterior pituitary contained predominantly chromophobic cells. Scattered among the chromophobes were a few cells with small dark nuclei and containing globules of red and purple PAS +ve material. These globules varied considerably in size and did not resemble the granules of the beta and delta cells.

Four of the experimental rats in the first long-term experiment developed pituitary neoplasms. These animals usually exhibited neurological signs, e.g. loss of balance and drooping eyelids, prior to the time of sacrifice. Pituitary neoplasms were discovered

in rats sacrificed 80, 112, 210 and 212 days after estradiol implantation. Neoplastic enlargement was also suspected in several other rats whose central nervous systems were unfortunately not examined at autopsy. The largest pituitary neoplasm weighed 378.1mg and was found in a rat sacrificed eighty days after estrogen treatment began.

Histologically these neoplasms appeared to be composed mainly of chromophobic cells, but also containing some alpha cells. The sinusoids were extremely constricted and there was much haemorrhage into the parenchyma of the pituitary. Many cells containing large globules of red and purple PAS +ve material were also present. The histology of these cells suggested that they were probably macrophages.

iii. Ovarian Weight and Histology. Following one month of estrogen treatment, the average weight of the ovaries was significantly ( $p < 0.05$  by t-test) less than that of the ovaries of the untreated control rats. An even greater ( $P < 0.001$  by t-test) decrease in weight was observed during the second month of hormone treatment. The ovarian weights of the experimental rats continued to drop slightly during the third and fourth months of estrogen treatment (Table IV, Fig. 43).

The most striking feature of the rats' ovaries following one month of estrogen-treatment was the presence of several large corpora lutea (Fig. 44b). Although the ovaries of the control rats contained more corpora lutea (Fig. 44a), those of the estrogen-treated animals were X2-X3 larger. Large corpora lutea persisted in some experimental ovaries for two to three months of estrogen treatment, however none could be found in the ovaries four months

after pellet implantation. By this time follicular growth was almost completely repressed (Fig. 44c) and the ovaries were so small that many of them were lost during automatic tissue processing.

The germinal epithelium of the rat ovary is continuous with the mesothelium lining the ovarian bursa, and both are derived from the same coelomic epithelium which is also invaginated to form the lining of the Müllerian duct. Following two months of estrogen treatment, the germinal epithelial cells, which are flattened in the controls, became rounded and showed some foci of proliferation. With continued hormone treatment the germinal epithelium became columnar and often formed papillae on the surface of the ovary. In extreme instances these papillae fused with the overlying bursal epithelium. A proliferation of the bursal mesothelium in one animal (Fig. 59a) closely resembled an ovarian papillary tumour.

Chronic inflammatory conditions of the oviduct occasionally involved the ovaries of estrogen-treated rats. In some instances this led to the formation of adhesions between the ovary and its bursa.

iv. Uterine Weight and Histology. The greatest increase in weight of the uteri of the rats implanted with pellets of estradiol occurred during the first month of hormone treatment (Table IV, Fig. 43). Although the average uterine weight of the experimental group was significantly ( $p < 0.05$  by t-test) greater than that of the controls throughout the four months, the weight changes observed in both groups during the second, third and fourth months were more or less equal.



The effects of long-term estrogen treatment on the uterus of the rat have been described in detail by several investigators (e.g. Bo, 1957; Korenchevsky and Hall, 1940; Zondek, 1941). All of the changes in uterine histology observed in the estrogen-treated rats in the present study have been discussed elsewhere, and therefore will be mentioned only briefly.

A keratinizing stratified squamous metaplasia of both the glandular and surface epithelium was the most consistent pathological finding in the uteri of the estrogen-treated rats of the present study. Cystic glandular hyperplasia, hypertrophy of the myometrium and varying degrees of inflammation including severe pyometra were also commonly observed in the treated rats.

Intraepithelial mucus inclusions, similar to those seen so frequently in the oviductal epithelium of estrogen-treated rats (see below), were observed in the uterine surface epithelium (Fig. 45) and also in the superficial layers of the stratified cervical epithelium.

Alcian Blue positive inclusions were also a striking feature of the cells of the only uterine neoplasm observed in the present study (Fig. 46). This neoplasm was found in the rat which had received estrogenic stimulation from a subcutaneous pellet for the longest period of time (i.e. 212 days). Mitoses could not be detected among the cells of this uterine growth. However areas of focal necrosis, anaplasia and invasion of the epithelial cells through the myometrium to the serosa strongly suggested that the neoplasm was malignant.

v. Mammary Gland Histology. All of the estrogen-treated rats examined in the first long-term study showed some degree of alveolar hyperplasia. In addition all of the mammary glands of the experimental animals showed evidence of milk production, which in some cases even amounted to huge milk-filled cysts that were visible to the naked eye.

Mammary carcinomas were found in two rats which were sacrificed following 105 and 210 days of estrogen-treatment. Another rat which was sacrificed following 112 days of estrogen treatment possessed a well-circumscribed mammary adenoma. The mammary glands of other estrogen-treated rats showed inflammatory changes.

#### Effect of Subcutaneous Implantation of Pellets of Estradiol on the Oviduct of the Rat

i. Oviductal Weight. Based entirely on weight changes, it would appear that of all the target organs of the rat examined in this study, the oviduct was the least responsive to the estrogenic stimulation. There were no significant differences observed between the oviductal weights of control and experimental groups throughout the four month period (Table IV, Fig. 43).

ii. Protein Synthesis and Microvillous Vesicles. The amount of protein synthesis occurring in the non-ciliated oviductal cells appeared to be increased in all of the estrogen-treated rats. This was suggested by an increase in the amount and dilatation of the rough endoplasmic reticulum. However the most noticeable morphological manifestation of the administration of estradiol to the intact rat that was observed during the early stages of hormone

treatment was the appearance in the oviduct of many intraepithelial mucus inclusions (Fig. 49).

The results in Part II of this report indicated that intraepithelial mucus inclusions would develop in the oviductal cells as early as six days after the initiation of hormone treatment. Following one month of estradiol treatment the mucus inclusions were found primarily in the junctura region of the oviduct. Oviducts of rats exposed to estradiol for longer periods of time showed inclusions throughout their length, although they were more numerous in the isthmus and junctura (Fig. 49) regions than in the preampullar and ampullar segments. Oviductal mucus inclusions were abundant following 2-3 months of estrogen treatment, but from thenceforth the numbers dropped (Fig. 49 c.f. Fig. 48).

Alcian Blue positive staining of the inclusions indicated a sulphated mucopolysaccharide composition. A similar staining of the luminal surface of control oviducts suggested that these inclusions contained the normal oviductal secretory product. This was also suggested by the fact that these inclusions only occurred in nonciliated cells.

Electron microscopic examination of similar mucus inclusions that developed in the oviduct of an intact immature rat following short-term estrogen treatment revealed them to be intracellular microvillous vesicles (Fig. 33). This was also true of the inclusions encountered in the mature rat oviduct following long-term estrogen treatment. The greater number of inclusions seen in this part of the study however meant that several stages in vesicle formation could be examined.

The flocculent substance contained within the vesicles was very similar to the material observed in the distended segments of rough endoplasmic reticulum (Fig. 33). This suggested the possibility of fusion of several cisternae to form one large vesicle, and indeed this has been observed (Fig. 50). Electron-dense secretory granules were seen only rarely near or within any of the microvillous vesicles. In addition, cells containing the microvillous vesicles possessed few, if any, normal secretory granules (Figs. 33, 51 & 52). This could indicate that these cells were incapable of condensing and packaging the secretory product for export in the normal manner.

There were fewer microvillous vesicles found in the oviducts of rats treated with estrogen for longer than three months than those examined in the earlier stages of hormone treatment. In many of the oviducts studied following the longest treatment periods the epithelium was distorted as a result of inflammatory conditions. However in some areas the epithelium was relatively unaffected and in these situations the non-ciliated cells contained an abundance of distended cisternae of rough endoplasmic reticulum and electron-dense granules scattered throughout the cytoplasm (Fig. 47-SG). During these later stages of estrogen treatment microvillous vesicles were only observed occasionally in the process of formation (Fig. 50).

iii. Inflammatory Changes and Bursal Cysts. As observed by the earlier investigators, inflammation of the oviduct was an almost invariable complication following long-term estrogen treatment in the rat. After one month of the hormone there was an influx of eosinophils into the serosal layer and after two months, several of

the oviducts exhibited a chronic perisalpingitis. With longer periods of estrogen treatment the number of inflamed oviducts increased and in some instances there was a secondary acute inflammation of the mucosa (Fig. 52).

Light microscopic examination of acutely inflamed oviducts revealed polymorphs emigrating through the epithelium (Fig. 53). Some of these polymorphs appeared to become trapped in the microvillous vesicles, thus forming 'intraepithelial abscesses' (Fig. 53). Large lysosome-type bodies found in the epithelial cells of the inflamed oviducts suggested that the oviductal cells were engulfing broken-down leukocytes and sequestering them in 'giant leukocytophagic lysosomes' (Fig. 54-GLL- see Appendix:Comment). The epithelial basal lamina of oviducts with acute inflammations of the mucosa was thickened in many places and often duplicated (Figs. 52 & 55).

If the acute inflammation of the mucosa persisted, pus collected in the lumen and a pyosalpinx developed. Resolution of the acute inflammation often resulted in a destruction of the mucosa and the blockage of the lumen with scar tissue.

Hydrosalpinx (Figs. 57 & 59) was often found as a sequela to severe acute inflammation of the oviduct. Adjacent oviductal segments blocked with scar tissue prevented proper drainage of the luminal fluid. The epithelium in cases of hydrosalpinx was either flattened (Fig. 57) or exhibited an unusual stratified metaplasia (Fig. 59c). The epithelial folds, if any remained, were rudimentary (Fig. 57).

The drainage of oviductal luminal fluid was sometimes prevented when there was no actual blockage of the oviduct itself. This was possibly the result of concomitant pathological processes in the



uterine horns of the estrogen-treated animals that led to the accumulation of luminal debris (e.g. desquamated keratinized cells). Back pressure of fluid into the closed ovarian bursa distended the lumen of the oviduct and flattened the mucosal folds (Fig. 48).

Several of the estrogen-treated rats showed varying degrees of distension of the bursae as a result of the increased fluid content. Damage to the ovary often led to bleeding into the bursa. Bursal cysts were present in one estrogen-treated rat. The walls of the bursae were toughened and fibrosed and the accumulation of bloody fluid had severely flattened the ovaries.

iv. Epithelial 'Proliferations'. Another common manifestation of long-term estrogen treatment in the oviductal epithelium of the rat was the development of foci of epithelial 'proliferation'. Unlike the intracellular microvillous vesicles which were formed as early as six days after the initiation of estrogen treatment, the epithelial 'proliferations' were much later in appearing following pellet implantation. They were found in one oviduct after two months of estrogen treatment, but were not common until three or four months.

The epithelial 'proliferations' generally took one of two very characteristic forms. The first type was a seemingly solid projection of cells usually arising from the tip of a mucosal fold (Fig. 58 a,b&c). The cytoplasm of the cells forming the projections often seemed denser than that of the surrounding epithelium. No core of lamina propria could be detected within the projections, and in some instances the outermost epithelial cells appeared to be undergoing necrosis (Fig. 58c).

The second kind of unusual epithelial configuration found in the oviducts from estrogen-treated rats was the appearance of an acinus of pale epithelial cells underlying one or more layers of cells typical of normal oviductal epithelium (Fig. 58d&e). In one acutely inflamed oviduct, the epithelium overlying one of these acinar configurations had apparently undergone a stratified squamous metaplasia (Fig. 56b).

These epithelial configurations, found only in the oviducts of the estrogen-treated rats, were designated as 'proliferations' primarily because this term has been used in the clinical literature for similar pathological changes found in the human oviduct and endometrium. It is important to note however that mitoses were never observed in the present study in association with the proliferative lesions. Mitoses were only rarely observed in acutely inflamed oviducts and were never observed in the oviductal epithelium of the control rats or those estrogen-treated rats that did not show acute inflammatory changes.

v. Adenomyosis and Reactions of the Oviductal Serosal Mesothelium to Long-Term Estrogen Treatment.

Adenomyosis was observed in the oviduct of one rat sacrificed 141 days after the implantation of an estradiol pellet (Fig. 60). A chronic salpingitis involved most of the layers of the oviduct of this rat and several of the mucosal folds had agglutinated. The resulting histological picture was suggestive of salpingitis isthmica nodosum.

The oviductal serosal mesothelium also showed striking reactions to the long-term estrogen treatment. Metaplasia into oviductal or endometrial-type of epithelium was commonly observed in the serosal

mesothelium of estrogen-treated rats (Figs. 59a&b, 61a,b &c). In one instance of oviductal metaplasia, the surface mesothelium also demonstrated the solid epithelial proliferations typically found in the oviductal epithelium following prolonged estrogenic stimulation (Fig. 61c).

Invagination of the surface mesothelium into the surrounding perisalpingeal tissues was also commonly seen in the estrogen-treated rats. Longitudinal sections of these invaginations appeared as deep clefts (Figs. 59a&b & 61a), whereas cross-sections of these invaginations suggested adenomyosis (Fig. 61b), a picture that was especially confusing when the invaginating mesothelium demonstrated oviductal or uterine metaplasia.

Extreme proliferative reactions of the mesothelium were also seen (Fig. 59a) and the picture presented in these cases closely resembled the proliferations observed in the ovarian germinal epithelium. The unusual histological organization of one mesothelial proliferation (Fig. 61d) was very reminiscent of a rare epithelial reaction which occurs in endometrial hyperplasia and which is described as a syncytial-like epithelial proliferation (see Novak and Woodruff, 1974, p.185). A similar epithelial reaction was also observed in the present study in a case of hydrosalpinx (Fig. 59c).

Although chronic perisalpingitis was observed in association with some of these alterations of the mesothelium, it was absent in other instances.

vi. Control Oviducts. The histology of the oviductal epithelium of the untreated rat has already been described in Part I of this report. None of the changes which were observed in the present

study in the oviducts of the estrogen-treated rats were detected in the oviducts of the control animals. In addition, several of the oviducts of the control rats contained eggs in cumulus masses, whereas none of the oviducts of the estrogen-treated rats did.

#### Effect of Subcutaneous Implantation of Pellets of Estradiol on the Oviductal Epithelium of the Rabbit

The study of Meissner, Sommers and Sherman (1971) is commonly quoted in discussions concerning the possible role of estrogen in carcinogenesis. Endometrial carcinomas developed in six of the eighteen rabbits treated with stilbesterol in their long-term estrogen experiments, whereas no malignancies were found in the control group.

No carcinomas were found in the uteri of the estrogen-treated rabbits in the present study. However the benign reactions of the ovaries and uteri of these rabbits were virtually the same as those described in detail by Meissner and his colleagues (i.e. cystic glandular hyperplasia of the endometrium, leiomyomas of the myometrium, papillary growths of the germinal epithelium and excessive luteinization of the ovarian stroma). For this reason only those abnormalities found in the oviduct following long-term estrogen treatment will be described in the present report.

1. Effects on Oviductal Secretory Cells. The cytoplasm of the secretory cells in the oviducts of rabbits exposed to prolonged estrogenic stimulation was bulging with granules and cisternae of rough endoplasmic reticulum that were grossly distended with a flocculent material (Fig. 62). Discharge of secretion or granules

into the oviductal lumen was not observed on a large scale, which was also true of the control estrus rabbits (see Part I).

In many of the estrogen-treated rabbits however, the basal cells membranes of the engorged secretory cells had apparently broken down, with a subsequent leakage of secretion and cellular debris into the intercellular space. Some of the flocculent material which originally was contained within the distended segments of endoplasmic reticulum was observed to pass through gaps in the basal lamina and into the lamina propria (Fig. 63). In one animal the leakage of 'secretory' material was so pronounced that the accumulation of this material in the subepithelial tissues appeared at the light microscope level as 'lakes' of secretion separating the epithelial cells and the connective tissue of the lamina propria.

ii. Hyperplastic Changes. The rabbit oviduct did not display 'proliferative' changes similar to those characteristic of the estrogen-treated rat oviduct. Instead, prolonged estrogenic stimulation led to a more diffuse type of hyperplastic change in the oviduct. That is, the epithelium exhibited tufting of the cells and nuclear crowding, and in general the folds were more 'adenomatous' than usual (Fig. 64, c.f. Fig. 3a). There also appeared to be a large increase in the number of ciliary vacuoles present in the epithelium of estrogen-treated rabbits. However whether this was related to the general hyperplasia cannot be determined.

iii. Viral-induced Salpingitis. Salpingitis affected the oviducts of three of the five estrogen-treated rabbits in the present study. Although estrogen-treatment commonly resulted in a peri-



salpingitis in the rat oviduct, the mucosa was the site of inflammatory change in the rabbit oviduct. The epithelium and lamina propria in these oviducts were heavily infiltrated with small lymphocytes.

Hyperplastic changes were also present in the epithelium of infected oviducts (Fig. 65), however because of the presence of inflammation the hyperplasia could not be directly attributed to the estrogen treatment.

Ultrastructural examination of the hyperplastic areas in one of the inflamed oviducts revealed an extremely confusing picture (Fig. 66). Cytoplasmic inclusions observed in several of the epithelial cells in these regions looked, at the light microscope level (Fig. 65b&c-I), very similar to the intracellular microvillous vesicles seen in the oviductal epithelium of the estrogen-treated rats. However electron microscope examination showed that these inclusions were found in cells which did not possess the cytoplasmic characteristics of the rabbit oviductal nonciliated cells.

In addition to the large vacuolar inclusions, small vacuoles resembling secondary lysosomes suggested that these 'foamy' cells might be intraepithelial macrophages. The unit membranes of these cells were hard to define and in some instances (Fig. 66b) appeared to be breaking down. Several of the large 'foamy' cells seemed to be multinucleated (Figs. 65a & 66b-MNC).

Intraepithelial viral particles were observed in the two oviducts demonstrating mucosal inflammation that were examined at the ultrastructural level. These viral particles were only found in the cytoplasm of the ciliated cells of the epithelium

(Figs. 67 & 68a).

Viral infection did not appear to cause cell death, but it did result in the destruction of the cilia (Figs. 67 & 68d). Debris accumulated in crypts of the rabbit oviductal epithelium which were lined by ciliated cells containing viral particles. This suggested that the cilia could be rendered inactive by the presence of the virus prior to the appearance of any morphological damage.

The viral particles averaged approximately 70nm in diameter and sections of the particles suggested a cubical symmetry. They appeared as aggregations in the cytoplasm of the ciliated cells, but intranuclear inclusions were never encountered. Among the clusters of membrane-bound electron-dense particles were aggregates of a slightly less dense material (Fig. 68a&b-arrows). These clusters of electron-dense material may represent the contents of viral particles which have shed their protein coat.

It could not be determined precisely from the morphology of the infected cells whether the viral particles were undergoing replication. Large fibrous inclusions were often present in the cytoplasm (Fig. 68c) and broken down basal bodies and what appeared to be small viral particles could be detected within these inclusions. Occasionally viral particles were observed in association with the surface of degenerating cilia (Fig. 68d). In these instances it could not be determined whether this represented a 'budding' of viral particles from the degenerating cilium or the attachment of virus particles to the cell membrane prior to their entry into the cell.

iv. Adenomyosis and Neoplasia. Adenomyosis was found in the oviduct of one rabbit sacrificed 355 days after estradiol pellet implantation (Fig. 69). There were no inflammatory changes associated with this condition, c.f. the case of adenomyosis seen in the oviduct of an estrogen-treated rat (Fig. 60).

Although no endometrial carcinomas developed in the estrogen-treated rabbits in the present study (or at least none detected by extensive macroscopic examination and routine histological examination of selected sections), there was one case of what appeared to be a primary oviductal carcinoma. This was detected during the histological examination of the oviduct of a rabbit which had been treated with estradiol for 291 days.

The lesion was located in the fimbria and examination of the rest of the oviduct did not reveal any other neoplastic foci. Small blocks had been taken from the contralateral oviduct for electron microscopic study and therefore widespread histological examination was only performed on the oviduct containing the neoplasm. Neither of the ovaries of this rabbit showed any signs of malignancy, although both ovaries possessed surface papillary formations (Fig. 72).

Several features of the fimbrial neoplasm suggested a malignant nature. A direct transition from typical columnar oviductal epithelium was observed (Fig. 70b). Solid clusters of neoplastic epithelial cells projected into the subepithelial tissues and in several places invaded the overlying epithelium (Fig. 70d).

Mitoses, however, were not observed. This suggested a low-grade malignancy, and the well-circumscribed nature of the lesion supported this conclusion.

THE EFFECTS OF LONG-TERM ESTROGEN ADMINISTRATION ON THE EPITHELIUM  
OF THE RAT AND RABBIT OVIDUCTS:DISCUSSION

Effects of Long-Term Estrogen Administration on the Hypothalamico-  
Pituitary-Ovarian Axis

i. Mature Rat. Long-term estrogen treatment had profound effects on the hypothalamico-pituitary-ovarian axis of the rat. These effects were reflected in morphological changes observed in the anterior pituitary and the ovaries of the treated animals. They were also ultimately reflected in changes in the organs that are under the hormonal control of the ovary and pituitary.

The interrelationships among the three components of this axis are complex, and not completely understood. All three components (i.e. hypothalamus, anterior pituitary and ovary) are target organs for estrogen action. In assigning causes to certain observable effects it is difficult therefore to decide whether they are the result of the direct action of estradiol on these organs, or a result of an estrogen-stimulated hormonal secretion from one of the other components of the system.

This study focused on the morphological changes elicited in the oviductal epithelium by long-term estrogen treatment. A detailed examination of the effects of this treatment on the whole animal was beyond the scope of the project. However certain changes in the hypothalamico-pituitary-ovarian axis ultimately affected the oviductal epithelium, and it is for this reason that these changes are being discussed.

In Part II of this study, the low dosage of estrogen (i.e. 1 $\mu$ g/day) administered to the immature rats was suspected of having

a facilitatory effect on gonadotropin release. In the long-term experiment however the larger quantities of exogenous estrogen (i.e. approximately 75 $\mu$ g/day; see Materials and Methods p.43) and the extended period of administration appeared to have an inhibitory effect on the gonadotropes. After one month of estrogen treatment the anterior pituitaries of the rats contained virtually no delta (gonadotrope) cells. This was also observed by Casas and Chang (1970) who were studying the effects of subcutaneous implantation of silastic tubing containing estradiol-benzoate (absorbed at a rate of 13.9 $\mu$ g/day). It is generally agreed (see Greep, 1961) that moderate to high dosages of estradiol administered to intact rats results in an inhibition of FSH and LH secretion and release.

At the same time (i.e. one month after pellet implantation) there was a noticeable increase in the alpha cell population of the anterior pituitary, an effect also observed by Casas and Chang (1970). In the anterior pituitary of the rat there are two types of cells found in the alpha cell population (also described as acidophils in some reports--see Purves, 1961&1966). These are the somatotropes (growth-hormone secreting cells) and the luteotropes or mammotropes (prolactin-secreting cells). Experimental evidence suggests that it is the prolactin-secreting cells which are involved in the estrogen-induced rise in alpha cells.

In the past, several investigators noticed that the administration of estrogen to intact rats resulted in an increase in the size of the ovarian corpora lutea and a prolongation of their

functional lifespan (Selye, Collip and Thomson, 1935; Wolfe, 1935). This was also observed in the present study. Recent evidence suggests that estrogen inhibits the hypothalamic prolactin-inhibiting-factor (PIF). With the cessation of hypothalamic inhibition, the prolactin-secreting cells of the anterior pituitary are free to proliferate, and to secrete and release prolactin (see Neumann et al., 1974). As prolactin is luteotropic in the rat, the increased release of this hormone explains the state of 'pseudopregnancy' observed in the estrogen-treated animals.

There was a considerable degree of variability observed in the length of time that large corpora lutea persisted in the ovaries of the experimental animals. Few corpora lutea remained after three months of estrogen treatment and none were present after four months. The considerable atrophy observed in the ovaries at this time reflected the lack of gonadotropic stimulation.

The factors responsible for the eventual luteolysis were not clear. Prolactin secretion and release continued however, as indicated by its effects on the mammary glands of the rat. All of the estrogen-treated rats in the first long-term study showed acinar proliferation and milk secretion. Huge milk filled cysts were found in the mammary glands of some of the rats, and one adenomatous and two malignant growths were found among the rats treated with estrogen for the longest periods of time.

In past experiments, enlarged pituitaries or frank pituitary adenomas were a fairly constant finding in rats bearing estrogen-induced mammary tumours (Cutts, 1966). In the present study, pituitary weight steadily increased with estrogen treatment and



although initially a rise in alpha cells was observed, after three months of estrogen the gland appeared to be composed primarily of chromophobic cells. This was also true of the pituitary tumours that developed.

Ultrastructural examination of estrogen-induced pituitary tumours reveals that they are composed primarily of hyperactive alpha cells (Attramadal et al., 1974). These cells contain large amounts of endoplasmic reticulum and Golgi saccules but few granules, which would account for the 'chromophobic' appearance of the cells at the light microscopic level. The increased release of prolactin by the cells is paralleled by high plasma levels of this hormone. It is this sustained secretion of prolactin that is now believed to be responsible for malignant changes in the breast, rather than a direct action of estrogen on the mammary gland (Beuving and Bern, 1972; Meites, 1972).

The conclusions of the present study were based on the supposition that the changes observed in the hypothalamico-pituitary-ovarian axis following estrogen implantation meant that the organs of the reproductive tract of the rat were receiving a biphasic type of hormonal stimulation. That is, during the first 1-2 months of treatment, the reproductive tissues were being subjected to a considerable progestational stimulation in addition to the exogenous estrogen. Following regression of the luteal tissue it was assumed that the main source of hormonal stimulation came from the pellet of estradiol.

Confirmation of this supposition could have been obtained from a regular analysis of the plasma steroid levels. A sincere

attempt was made to organize such an analysis, but the expense involved proved to be prohibitive. Therefore, the possibility of non-ovarian sources of endogenous steroid hormones (i.e. estrogen and progesterone) has been ignored. There has also been no attempt to relate the changes observed in the oviductal epithelium to the increase in prolactin secretion. As yet there has been no established role for this hormone in the physiological regulation of oviductal function.

ii. Mature Rabbit. There is little experimental evidence available to suggest what happens to the hypothalamico-pituitary-ovarian axis of the rabbit chronically treated with estrogen. A repression in follicular growth was observed in the present and other studies (Mazer, Israel and Alpers, 1936; Meissner, Sommers and Sherman, 1957). At the same time a considerable increase occurs in the amount of interstitial tissue found in the ovary (Ibid.).

Experimental evidence suggests that in the normal (i.e. untreated) rabbit, the interstitial tissue produces predominantly  $20\alpha$ -hydroxy-pregne-4-en-3-one ( $20\alpha$ -OH) (Eaton and Hilliard, 1971). This is a weak progestin which is not capable of maintaining pregnancy in the rabbit in the absence of a corpus luteum. Its function is not clear, however it has been suggested that  $20\alpha$ -OH might act as an anti-progestin by competing with progesterone for cytoplasmic binding sites (Perry, 1971).

The neural stimulus elicited during mating is generally required in order for ovulation to occur in the rabbit, and estrogen treatment will induce ovulation in only very exceptional cases (Everett, 1961). There were no corpora lutea found in the

ovaries of the estrogen-treated rabbits in the present study, so it can be assumed that the estrogen released by the pellet was unopposed by progesterone secretion. In the absence of corroborating plasma steroid assays it cannot be known for certain whether the large amount of interstitial tissue observed in the ovaries of the estrogen-treated rabbits was actively secreting  $20\alpha$ -OH. Nor is it known whether this steroid might affect estrogen action within the oviductal epithelium. Present evidence indicated that the progestational steroid which is taken up and specifically bound by target tissues appears to be progesterone itself (Jensen and DeSombre, 1972).

#### Exogenous Estrogen and Oviductal Infections

i. Rat Oviduct. The estrogen-treated rats in the present study developed oviductal inflammations almost without exception. Two months after pellet implantation the oviducts of several of the rats were affected by a chronic perisalpingitis. Inflammatory reactions of this sort were encountered with greater frequency as the duration of estrogen treatment increased. Secondary acute inflammations of the mucosa and pyosalpinx developed after several months, and the oviducts of rats sacrificed after the longer periods of estrogen treatment often showed sequelae of inflammatory processes, e.g. hydrosalpinx, mucosal destruction and blockage of the lumen with scar tissue.

In human beings, non-granulomatous salpingitis develops by one of three processes (Woodruff and Pauerstein, 1969). An endosalpingitis is generally the result of an ascending infection

(usually gonococcal) transmitted via the cervix and the endometrium. On the other hand pyogenic organisms from puerperal or post-traumatic endometritis are frequently disseminated through the myometrial lymphatic and vascular channels, thus resulting in a perisalpingitis.

Perisalpingitis may also develop as a result of direct spread from adjacent gut inflammations. However these were not encountered regularly in the estrogen-treated rats, and when bowel abscesses were present, they had quite obviously arisen from adherence of the gut to large pyometrial masses. It is most likely therefore that the perisalpingitis commonly seen in the estrogen-treated rats arose from lymphatic or vascular spread of an acute or sub-acute endometritis.

At this point it is important to remember that during the initial 1-2 months of estrogen treatment, the reproductive tracts of the rats were also being subjected to a significant progestational stimulation (see p. 141). Ovarian hormones have been observed to influence the course of reproductive tract bacterial infections and several workers have stressed the correlation between the persistence of a corpus luteum and the development of uterine infection (Broome, Lamming and Woodbine, 1959; King, 1973).

In 1937, Weinstein, Gardner and Allen tested the uteri of mice for the presence of bacteria and examined the possibility that estrogenic hormones could induce changes in this organ that would result in bacterial invasion. They found bacteria in the uterus of only two of the forty-five control mice, and both of these mice showed 'postestrus' vaginal smears. They then tested the effects

of a single subcutaneous injection of estrogen (50µg hydroxy-estrin benzoate). Two days after the injection bacteria were detected in the uteri of some mice and by seven and fourteen days after the injection most of the experimental mice had intra-uterine bacteria. Observations on mice whose uterine horns had been ligated indicated that the bacterial invasion had occurred by direct extension through the cervix, and cultures obtained from uterine and vaginal smears yielded similar organisms.

Weinstein and his colleagues assumed that some direct effect of estrogen on the uterus had resulted in the bacterial invasion. It seems more likely however that the estrogen treatment had resulted in a prolongation of corpus luteum function, and that the increase in progesterone release had produced the conditions which allowed for bacterial penetration into the uterus. The regulation of ovarian hormonal output by the hypothalamus and pituitary is very similar in the rat and the mouse (Perry, 1971). The manner in which exogenous estrogen results in increased prolactin secretion (which is luteotropic in both rats and mice) has already been discussed. Evidence has also been cited which indicates that progesterone, even when secreted at a relatively low level, will cause a physical relaxation of the cervixes of the rat (see Discussion-Part II).

All of the evidence taken together therefore suggests that the bacterial invasion of the uterine horns which Weinstein and his colleagues observed in their estrogen-treated mice, and which probably produced the endometritis that eventually spread to the oviducts of the estrogen-treated rats in the present study,

was due to an estrogen-induced increase in progesterone secretion. Supporting this hypothesis is the fact that the only control mice found to have uterine bacteria in the study of Weinstein and his colleagues were mice which also exhibited progestational (i.e. 'post-estrus') vaginal smears.

There is also experimental evidence that the conditions induced in the endometrium by progesterone may hamper the passage of leukocytes through the tissues to the lumen, and that secretions of the endometrium under the influence of progesterone might even favour the growth and survival of bacteria (Heap, Robinson and Lamming, 1962). This could explain why the reproductive tract infections in the estrogen-treated rats progress so far and appear to be so highly virulent.

ii. Rabbit Oviduct. Mucosal inflammatory changes were observed at the light microscope level in three of the five estrogen-treated rabbits. Electron microscopic examination of the oviductal epithelium of two of the three rabbits revealed that these animals appeared to be suffering from a viral-induced salpingitis. (Note: No ultrastructural examination was performed on the oviduct of the third rabbit, but the light microscope picture was the same as the other two animals.)

There are four major criteria generally used in the classification of viruses: nature of the viral nucleic acid, structure of the viral particle, the presence or absence of a membranous envelope surrounding the protein coat of the virus, and the dimensions of the particle. The viral particles observed in the rabbit oviductal ciliated cells were polyhedral in shape, measured



approximately 70nm in diameter and did not possess a membranous envelope. Although the type of nucleic acid present within the viral particles could not be determined from the morphological examination, there is only one class of known viruses that is characterized by the three features mentioned above. This group of viruses is the double-stranded RNA viruses, i.e. the reoviruses.

One of the most interesting observations in the present study was that virus particles were only detected within ciliated cells and in many of the infected cells the cilia appeared to be degenerating. At present there is no mention in the literature of a particular affinity between reoviruses and ciliated cells.

However in those cells (mainly cell-culture systems) in which reovirus replication has been examined morphologically, a close association between foci of virus replication and cytoplasmic microtubules (particularly the mitotic spindle) has constantly been observed (Dales, 1975; Kohler and Spendlove, 1974; Shatkin, 1968).

Using fluorescent-labeled or ferritin-conjugated antibodies raised against reovirus particles, viral antigens have been observed coating cytoplasmic microtubules and the mitotic spindle apparatus (Dales, 1975; Kohler and Spendlove, 1974). That this association between viral proteins and the microtubules might cause a disturbance of the functioning of the microtubules is indicated by the fact that abnormal mitotic figures were observed in virus-infected fibroblasts (L-cell cultures; Dales, 1975).

Thus the discovery of fragments of broken down ciliary axonemes and basal bodies in the cells containing viral particles in the rabbit oviduct was particularly interesting. At present only the morphological association between reovirus nucleic

acid and protein and cytoplasmic microtubules has been documented. Dales (1975) has postulated that the transcription and replication of the viral genome occurs on the microtubules. It has been observed (see Shatkin, 1968) that virus replication is not dependent on mitotic spindle formation, for treatment of infected cells with colchicine does not reduce the yield of infectious virus. This however is not evidence against the possibility that it is the microtubule protein that is required.

Another interesting observation was the presence of large fibrous inclusions in infected cells surrounding what appeared to be fragments of basal bodies and cilia. If these fragments were coated with viral antigens this 'fibrous' reaction might be a specific intracellular response to the presence of the pathogen. Taylor-Robinson and his colleagues (Taylor-Robinson et al., 1974) examined with the electron microscope the interaction of Neisseria gonorrhoeae with the human oviduct in organ culture. In some of their photomicrographs intracellular bacteria appear to be surrounded by bundles of fibres in a manner similar to that observed in the present study.

The mechanism by which tissues react to and recover from viral infection is not clearly understood. Viral invasion of a cell is followed by the production of interferon, a protein synthesized by the host cell which limits virus growth. As cells become infected the amount of interferon produced increases and so less virus is synthesized and fewer new cells become infected. Interferon production is only partly responsible for recovery however, for children with deficiencies in cell-mediated immune responses have difficulty in coping with viral diseases.

It is not exactly clear how cell-mediated immunity contributes to the recovery from viral infections. It is suspected that the process involves the attraction of macrophages to areas of viral infection by 'lymphokines' released from thymic-dependent lymphocytes which have been stimulated by contact with viral antigens. These macrophages are subsequently activated and stimulated to produce interferon. They may also phagocytize and kill the viruses (Roitt, 1974). It is also possible that the macrophages modify the ingested antigen in such a way as to make it more strongly 'immunogenic' to the responsive lymphoid cells.

It is generally agreed that the inflammatory reaction to the presence of viruses is characterized by a local lymphocytosis. In the rabbit oviduct there was a profound invasion of the epithelium by small lymphocytes. Also present within the epithelium were large 'foamy' cells. These cells aggravated the ultrastructure of areas which in light micrographs appeared as foci of proliferation.

Small vacuoles resembling secondary lysosomes suggested that the 'foamy' cells might be macrophages. They were often found in close proximity to the small lymphocytes. In several instances the unit membranes of the foamy cells were hard to define and in some cases arrangements of cells suggested giant-cell formation. Multinucleate cells arising through the fusion of viral-infected macrophages is a known phenomenon. Viruses will also cause fusion of epithelial cells (syncytium formation), however the information available suggests that this response is not characteristic of epithelial cells infected with reoviruses.

In summary, the inflammatory response observed in the rabbit oviduct, which was characterized by the presence within the

epithelium of large numbers of small lymphocytes and cells presumed to be macrophages was a result of a virus infection. The morphology of these particles and the fact that the pathological effects were confined to the cytoplasm suggested that the virus belonged to the reovirus class. Only the ciliated cells contained viral particles and the only visible pathological effect was a breakdown of the basal bodies and ciliary axonemes.

As ciliated cell death did not occur on a large scale it appears that these microbes were non-virulent as regards the oviductal epithelium. Reoviruses are believed to cause mild respiratory infections in man and it would be interesting to know whether this was a result of a comparable effect on the ciliated cells of the respiratory tract.

Within the scope of present knowledge, there does not seem to be any justification for correlating the viral infections with the estrogen treatment. Too little is known about how exogenous estrogen affects the intact rabbit's ability to cope with disease, although it has been observed that the efficiency of the estrus doe in dealing with intrauterine bacterial infection is greater than that of the pseudopregnant rabbit (Heap, Robinson and Lamming, 1962). Meissner, Sommers and Sherman (1957) did find inflammatory changes in the oviducts of their stilbesterol-treated rabbits, but they did not specify the nature of the inflammatory change.

In the present study the estrogen-treated rabbits and the control rabbits were housed in separate rooms in the animal house, and were not necessarily obtained from the same source. Nor were all the control and experimental rabbits present in the animal

house during the same time period, which means that it is quite likely that not all of the animals were exposed to the same 'microbial' environment.

Nevertheless, the chance encounter of the viral-induced salpingitis provided an excellent opportunity to examine the reaction of the oviductal epithelium to an abnormal stimulation. The few observations that were made on the interaction between cell and pathogen added an extra dimension to the study of the oviductal epithelium, and somehow made this tissue seem more 'alive'.

#### Effect of Long-Term Estrogen Treatment on the Oviductal Epithelium

##### i. Rat Oviduct- Early Effects (i.e. after 1-2 months) of Estrogen Treatment.

It has already been described how the subcutaneous implantation of a pellet of estradiol affects the hypothalamico-pituitary-ovarian axis of the rat. The prolongation of luteal function as a result of the estrogenic inhibition of the hypothalamic prolactin-inhibiting-factor ultimately meant that, as long as the corpora lutea persisted, the oviductal epithelium was being subjected to a progestational stimulation in addition to the estrogenic stimulation. The factor responsible for the eventual atrophy of the corpora lutea in the face of continuing prolactin secretion is still unknown. In the present study, corpora lutea were found to persist in the ovaries of the estrogen-treated rats for approximately 1-2 months.

It is not entirely clear how progesterone acts within target cells or how it modifies the intracellular effects of estradiol. In some target cells progesterone stimulates protein synthesis in a manner analogous to that previously described for estradiol

(see Introduction). Although estrogen will cause the differentiation of goblet cells in the chick oviduct, only progesterone can provoke the synthesis of avidin by these cells (O'Malley et al., 1974).

Estrogen administration will cause the synthesis of four egg white proteins (ovalbumin, lysozyme, conalbumin and ovomucoid) by the tubular gland cells of the chick oviduct. Palmiter and Smith (1973) observed however that a combination of estrogen and progesterone would greatly increase the yield of conalbumin and ovomucoid. Because the mRNA sequences coding for these two proteins were present in twice to three times the amount found in the oviducts of chicks treated with estrogen alone, these workers postulated that there were multiple regulatory sites for the genes coding for conalbumin and ovomucoid. These regulatory sites, when activated by one or more different steroid-receptor units, could therefore produce independent or co-operative effects on mRNA transcription. Thus Palmiter and Smith have provided one possible explanation for synergistic effects observed following the combined administration of estrogen and progesterone.

Progesterone has also been observed to antagonize many processes stimulated by estradiol (see Part II, p.109). Although estrogen and progesterone do not compete for the same cytoplasmic receptor protein, there is evidence that progesterone interferes at some stage with the translocation and nuclear binding of the estrogen-receptor complex (Brenner, Resko and West, 1974; Taylor, 1974; Trams et al., 1973). In estrogen-dependent tissues, the 'antagonistic' effects that are observed following progesterone



administration would thus actually be due to estrogen deprivation.

With the absence of hormone-treated ovariectomized experimental rats it is impossible to state with any degree of certainty whether the changes observed in the oviductal epithelium in the present study were due to the action of estradiol or progesterone or occurred as a result of the combined action of the two hormones. Some clues however to the hormonal factors responsible for the changes observed in the long-term study can be obtained from the results of the short-term study described in Part II of this report.

The oviductal epithelium of the immature rat is being stimulated by low levels of endogenous estradiol. Exogenous estrogen increased the amount of protein synthesis occurring in the cells. This was suggested by a hypertrophy of the nucleolus (indicating heightened ribonucleoprotein synthesis) and a relative increase in cytoplasmic rough endoplasmic reticulum.

After several days of estrogen treatment, gonadotropins released from the anterior pituitary of the immature rat stimulated ovarian secretion of progesterone (see Part II-Discussion). The cell and nuclear volumes of the oviductal epithelial cells returned to within control values, suggesting that the endogenous progesterone had antagonized the estrogen-induced increases in these parameters.

Progesterone also inhibited ribonucleoprotein synthesis, as indicated by a decrease in nucleolar size. There was still a considerable amount of rough endoplasmic reticulum however, and after an estimated three days of combined estrogen and progesterone stimulation the cisternae of RER were widely dilated.. In addition, several microvillous vesicles were found within the isthmic

epithelial cells.

A similar picture was presented by the oviductal epithelium of the mature rats treated for 1-2 months with estrogen. Intracellular microvillous vesicles were abundant, and from the ultrastructure it appeared as if the inclusions developed as a result of the fusion of distended cisternae of RER. However the contents of the vesicles stained with Alcian Blue, suggesting a sulphated mucopolysaccharide composition similar to the normal oviductal secretory product. The presence of sulphated sugars responsible for the Alcian Blue staining within the vacuoles implied that the Golgi apparatus was involved in the vesicle formation, although the morphological examination had not suggested this.

There was a paucity of secretory granules in the oviductal cells containing microvillous vesicles. This suggested that these cells were unable to condense the secretory product into export form (i.e. granules) and as a result, the diluted mucopolysaccharide accumulated in the large intracellular vesicles.

The manner in which progesterone might prevent the efficient secretory process of the oviductal cells is not clear. Nyak and Zimmerman (1971) observed the effects of estrogen and progesterone on the oviductal epithelium of the ovariectomized gilt and it is interesting to note that the administration of estrogen alone resulted in an increase in endoplasmic reticulum and Golgi saccules and the formation of many secretory granules, whereas progesterone alone could only effect an increase in the number of ribosomes and endoplasmic reticulum in the nonciliated cells of the oviduct.

In all of the previous studies of the effects of prolonged estrogen treatment on the reproductive tract of the rat, there is only one mention of intraepithelial mucus inclusions. Arias-Stella (1955) found similar changes in the uterine epithelium of intact rats treated with estradiol and chorionic gonadotropins for 18-20 days. He believed that the vacuoles resulted from an overproduction and faulty secretion of mucus and therefore he called them "intraepithelial retention cysts". The ovaries of the rats treated with estrogen and chorionic gonadotropins contained huge corpora lutea, and using ovariectomized rats Arias-Stella was able to induce the inclusions with combined administration of estrogen and progesterone, but not with estrogen alone. (Arias-Stella did not find uterine 'retention cysts' in his intact rats treated with estrogen alone. However the ovaries of these rats did not contain the large corpora lutea commonly found in the present study after one month of estrogen treatment.)

Commenting on the lack of similar findings in other experiments where rats were treated with a combination of estrogen and progesterone, Arias-Stella concluded that an important factor in the eventual effect observed following the administration of a combination of hormones was the relative amounts of estrogen and progesterone (i.e. the E/P ratio) stimulating the reproductive tracts. Intraepithelial mucus inclusions were observed occasionally in the surface epithelium of the uteri of the estrogen-treated rats in the present study, but they were far less abundant than the amount found in Arias-Stella's rats.

It is puzzling why the intraepithelial mucus inclusions should develop in the uterine epithelium in the first place. The surface

epithelium of the rodent uterus consists of uniform columnar cells. Electron microscopic examination reveals surface microvilli, rough endoplasmic reticulum, Golgi apparatus and small apical vesicles, but no secretory granules comparable to those found in the oviductal epithelium (Nilsson, 1958c). The apical vesicles of these cells have been implicated in endocytosis rather than in a secretory process (Parr and Parr, 1974).

It was not surprising to discover these inclusions in the oviductal cells because the nonciliated cells of the oviduct, and especially those containing granules, actively take up radioactive sulphur (presumably to be incorporated into acid mucopolysaccharides) (Boström and Odeblad, 1952). However only very small amounts of radioactive sulphur can be detected by autoradioactive means within the surface and glandular epithelial cells of the rat uterus (Ibid.).

One explanation for this phenomenon (which will be discussed in greater detail later) is that the cells containing the mucus inclusions in the surface epithelium of the uterus had undergone a metaplastic transformation into an oviductal type of cell prior to the development of the mucus inclusions. Evidence will be discussed later which suggests that hormonal imbalances can actually provoke metaplastic alterations in the epithelium of the reproductive tract. The fact that Arias-Stella (1955) found many more mucus inclusions in the uterine epithelium of his rats than was found in the present study could indicate that his experimental procedures resulted in a more radical alteration in hormone balance and thus in a more widespread metaplasia.

ii. Rat Oviduct- Late Effects (i.e. after two months and longer) of Estrogen Treatment. The majority of the corpora lutea found in the estrogen-treated rats only persisted for 1-2 months, and the ovaries of a large percentage of the rats sacrificed after three months of estrogen treatment no longer contained corpora lutea. Hence in the majority of rats the estrogen being absorbed from the subcutaneous pellet was unopposed by progesterone a few months after pellet implantation. This probably accounts for the dwindling number of microvillous vesicles observed in the oviducts removed after the longer periods of hormone treatment.

It is also likely, therefore that the 'proliferative' changes observed in the oviductal epithelium of rats sacrificed after longer than three months of hormone treatment were due to the unopposed estrogenic stimulation. This is also supported by the fact that in only one instance were proliferative nodules observed prior to three months, suggesting that the endogenous progesterone secreted by the enlarged corpora lutea prevented the development of these nodules.

Two types of histological organization were observed in the 'proliferative' lesions found in the rat oviductal epithelium following long-term estrogenic stimulation. Papillary structures composed of a solid projection of cells lacking any obvious stromal component were often seen arising from the surface of the epithelium. Similar configurations have been observed in the human oviduct (Moore and Enterline, 1975; Pauerstein and Woodruff, 1966), and Pauerstein and Woodruff observed the 'proliferative' changes were frequently seen with excessive exogenous or endogenous

estrogenic stimulation.

Pseudo-glandular changes were also seen in the oviductal epithelium of the estrogen-treated rats. Dougherty and Cotten (1964) described a case where an estrogen-secreting ovarian tumour was associated with proliferation and pseudostratification of the human oviductal mucosa. The oviductal epithelium in this instance also contained small, secondary gland-like configurations. Dougherty and Cotten observed that in spite of the obvious multiplication of the cells involved in the formation of these proliferative lesions, very few mitoses could be detected.

Although demonstrating considerable anaplasia at times, the rat proliferative lesions cannot be considered carcinoma-in-situ. By definition, the presence of mitoses is required for this term to be applicable (Pauerstein and Woodruff, 1966), and mitoses were never observed in association with these changes in the rat oviduct. The possibility exists however, that these lesions might indicate a premalignant change.

As yet definite premalignant lesions of the oviductal epithelium have not been established (Ibid.). However some of the epithelial alterations seen during the early stages of uterine and oviductal carcinogenesis closely resemble the changes observed in the present study. In 1900 Bullen (see Gray and Barnes, 1964) described the histological changes found in carcinoma arising from the surface epithelium of the uterus. At some distance from the malignant growth, epithelial changes consisting of cells totally devoid of supporting stroma could be seen. There is a striking similarity between the lesions observed in the present study and Cullen's diagrams of the uterine proliferative foci.



Anbrokh (1970) has made an extensive study of the histogenesis of oviductal carcinoma. He observed that in many cases where microcarcinomas or solitary neoplastic nodules were found, additional multicentric foci of atypical epithelial proliferations could be detected both near to and at a distance from the malignant changes.

Although estrogen has never been implicated in the etiology of oviductal carcinoma, persistent inflammation has been suspected as one factor responsible for malignant change (Anbrokh, 1970). In the present study mucosal inflammatory changes were absent in the majority of oviducts demonstrating proliferative lesions, which is further evidence that estrogen was responsible for these alterations. However other epithelial changes were observed in the oviducts of estrogen-treated rats in which salpingitis involved the mucosal layer. The presence of inflammatory changes in these instances meant that the factor provoking these alterations could not be isolated with certainty.

Adenomyosis and stratified squamous metaplasia of the epithelium were both seen in cases of oviductal mucosal inflammation. It has already been observed that perisalpingitis commonly develops a few months after estrogen implantation. However it cannot be determined with certainty whether in these cases adenomyosis (or stratified squamous metaplasia) developed before or after the secondary mucosal inflammation. The co-incidence of salpingitis and adenomyosis or similar changes which occur in the oviductal and uterine mesothelium (see below) has often been taken as an indication that these alterations occur in response to the inflammation. However there is also evidence that suggests that these

particular changes may predispose the oviduct to infection (Freakley et al., 1974).

In conclusion, the most significant change observed in the oviductal epithelium of the estrogen-treated rats following the regression of the corpora lutea was the development of 'proliferative' lesions. These were attributed to the effects of unopposed estrogenic stimulation of the oviductal cells. One case each of adenomyosis and stratified squamous metaplasia were also found following long-term estrogen treatment. However the co-incidence of mucosal inflammatory changes in these instances prevented the conclusive identification of estrogen as the causative agent.

iii. Rabbit Oviduct. The nonciliated cells of the oviductal epithelium of rabbits treated for long periods of time with estrogen were engorged with secretion. Although microvillous vesicles similar to those found in the oviducts of estrogen-treated rats did not develop, the basal half of the secretory cells of the rabbit appeared to consist of one huge distended cisterna of rough endoplasmic reticulum.

The morphological appearance suggested that, as in the rat, the oviductal cells of the estrogen-treated rabbit were unable to disgorge their contents. An inhibitory effect of progesterone on secretion release was suspected as the cause of the development of 'retention cysts' in the rat oviduct. However in the rabbit oviduct it is most likely that the estrogen treatment was solely responsible for this effect.

Greenwald (1958,1969) has studied the regulation of secretion release from the oviductal epithelium of the rabbit. He found that

although estrogen was necessary for the synthesis of secretory product by the epithelial cells, progesterone was responsible for the release of the granules. The administration of estrogen to rabbits after mating results in a drastic reduction in the layer of mucin deposited around the oviductal eggs, and histological examination of the oviducts of these animals reveals that the cells are still bulging with granules (c.f. controls whose oviductal cells release their granules during egg transport-Greenwald, 1969).

Prolonged retention of mucus appeared to have a deleterious effect on the rabbit oviductal cells. In several situations the basal cell membranes of the secretory cells seemed to have broken down, with a subsequent leakage of secretory material into the intercellular space. This material often spread to the lamina propria through gaps in the basal lamina, and accumulated in subepithelial 'lakes' of secretion which were very conspicuous in histological preparations.

Prolonged estrogenic stimulation resulted in a diffuse hyperplasia of the epithelium, which was also observed by Meissner, Sommers and Sherman (1957) in the oviducts of their stilbesterol-treated rabbits. These changes included nuclear crowding and cellular tufting and an increase in the 'adenomatous' appearance of the mucosal folds. In some areas the epithelium appeared to be stratified, but the invasion of lymphocytes and macrophages into areas of viral-infected epithelium confused the histological picture.

It was noted in Part I of this study that the epithelium of the rabbit oviduct dips down into pits between the mucosal folds.

In one of the estrogen-treated rabbits invagination of the epithelium had extended through the muscle layer, resulting in a histological picture typical of adenomyosis. No inflammatory changes could be detected in this region of the oviduct, thus leaving the estrogen treatment as the prime suspect for causative agent. Marcus (1961) has made a survey of the co-incidence of adenomyosis uteri and endometrial hyperplasia and endometrial carcinoma in the human subject, and concluded that the interrelationship among these three conditions suggested a common causative factor (which might be hormonal).

A lesion believed to be carcinoma developed in the fimbriated end of the oviduct of one of the estrogen-treated rabbits in the present study. Again there were no obvious signs of inflammation at the site of the lesion. This neoplasm demonstrated a solid, rather than a papillary or alveolar structure, which is usually considered to be rare as far as human oviductal carcinoma is concerned (Novak and Woodruff, 1974; Woodruff and Pauerstein, 1969). However in Anbrokh's (1970) survey of human oviductal carcinomas, 9.8% of his cases were of a solid form.

A conspicuous absence of mitoses, the prime evidence against a diagnosis of malignancy initially suggested that the lesion might be an adenomatoid tumour. This type of tumour arising from the serosal mesothelium accounts for the majority of benign growths in the human oviduct.

However the typical histological organization found in adenomatoid tumours (i.e. a proliferation of mesothelial-lined tubules- Pauerstein, Woodruff and Quinton, 1968; Salazar, Kanbour and Burgess, 1972) was not observed in the present instance.

Instead the cellular pattern consisted in many places of solid cords of anaplastic cells, although tubules lined with well-differentiated oviductal epithelium could be seen in certain areas. In addition, a direct transition from oviductal to neoplastic epithelium was found on the fimbria. Observations of normal fimbriae showed that the transition from oviductal epithelium to serosal mesothelium did not occur on the fimbria but rather on the wall of the oviduct proper (see Fig. 71), further evidence suggesting that the tumour had an epithelial, rather than a mesothelial, origin.

Inspection of the ovaries of this animal showed no malignant changes, although each ovary had large surface papillae, a response to long-term estrogenic stimulation also observed by Meissner, Sommers and Sherman (1957). Direct spread of tumour cells from the uterus seemed unlikely as the lesion was limited to the fimbriated end of the oviduct, and as mentioned before, a direct transition from normal to neoplastic epithelium had been found on the fimbria. In any case, the sections of the uterine horns of this rabbit which had been examined histologically had revealed no malignancies. All of the evidence seemed to indicate that this lesion was probably a carcinoma of low-grade malignancy (suggested by a scarcity of mitoses), and may represent the first oviductal neoplasm to be reported that had developed in an experimental animal undergoing long-term estrogen treatment.

#### Estrogen Treatment and Metaplasia

Novak and Woodruff (1974) designate metaplastic change which occurs in the oviduct as a 'heterotopic' alteration when

the metaplastic epithelium is representative of another region of the reproductive tract. They found the fact that this type of change also occurred in the endometrium and endocervix indicative of the multipotentiality of the Müllerian epithelium.

Metaplasia was commonly observed in the present study in the reproductive tracts of rats treated with estrogen. Stratified squamous keratinizing epithelium was often found replacing the columnar epithelium of the endometrium and the endocervix. In one instance a stratified squamous change was observed in the oviduct. Cells containing Alcian Blue positive mucus inclusions were observed occasionally in the uterine and cervical epithelium and as discussed previously, this was believed to indicate the replacement of uterine cells with oviductal elements.

In addition to the observations of the present study, there is clinical evidence that excess estrogen provokes 'heterotopic' change. Ciliated cells and areas of oviductal epithelium are commonly found in endometrial hyperplasia, a condition associated with hyperestrogenism (Novak and Woodruff, 1974; Schueller, 1973). Fruin and Tighe (1967) found that the incidence of oviductal metaplasia observed in the human uterus increased progressively as the endometrium passed from a simple proliferative state through a mild hyperplasia to a non-secreting cystic hyperplastic condition.

Hormonal imbalance may also be the cause of endometriosis (Novak and Woodruff, 1974). There are two main theories accounting for the development of ectopic functioning endometrial tissue. In 1921, Sampson (see Novak and Woodruff, 1974, p.518) first described this pathological entity and hypothesized that the condition arose when fragments of endometrium were 'regurgitated'



through the oviductal opening and subsequently implanted in the peritoneal cavity. The opposing theory is that the aberrant endometrium arises from an abnormal differentiation of the coelomic mesothelium lining the pelvic cavity. Although the 'implantation' theory is considered to be feasible in certain instances of endometriosis, the majority of cases are believed to represent 'abnormal' differentiation, especially since endometriosis has been discovered as far afield as in the pleural cavity (Irani et al., 1976).

In the human subject, the ovary is the prime site for the development of endometriosis, and Novak and Woodruff (1974) suggest that this is because the 'germinal' epithelium represents the original coelomic mesothelium which invaginates to form the Müllerian duct, and thus presumably retains its capacity for differentiation into a reproductive-type of epithelium. Oviductal and uterine epithelia were also observed replacing the mesothelium of the oviductal serosa and lining the ovarian bursa in the estrogen-treated rats of the present study. Whether the alterations seen in the bursal and serosal mesothelium represent metaplasia, or else a switch from an 'undifferentiated' stem cell tissue (e.g. primitive coelomic mesothelium) to a more highly differentiated functional tissue, is not clear.

The traditional concepts of differentiation have changed considerably during the past few years and recent experimental evidence does not substantiate the supposed rigidity of developmental pathways. The observations in the present study confirm the notion expressed by Novak and Woodruff and many others that the epithelial cells of the reproductive tract originally

derived from the Müllerian duct retain the capacity to 'differentiate' along several lines depending upon the type of stimulus they receive. This includes the capacity to produce a stratified squamous response, which is the commonest type of metaplasia observed in the endometrium and endocervix of estrogen-treated rats.

For several years the accepted explanation for this phenomenon was Zuckerman's 1940 hypothesis (see Raynaud, 1962) that the stratified squamous epithelium arose from cells derived from the urogenital sinus which had migrated upwards into the cervix and uterus. However Gitlin(1954) doubted this hypothesis when he found patches of stratified squamous epithelium at a considerable distance from the squamo-columnar junction in the uteri of estrogen-treated rats. In addition, several recent embryological studies have shown convincingly that the cells of the caudal end of the Müllerian duct are capable of giving rise to the stratified squamous epithelium of part of the vagina (Bulmer, 1964; Forsberg, 1973), thus including this type of response in the repertoire of functional pathways available to the cells of the reproductive tract.

There are two possible explanations for the presence of heterotopic alterations in the epithelium of the 'normal' reproductive tract. One is that there is a mixed population of epigenotypes (i.e. stem cells-see Nevo, Weisman and Sadé, 1975) distributed along the reproductive tract, each of which is programmed to give rise to a single type of daughter cell and that metaplastic elements arise from 'displaced' epigenotypes. Or else cells of the Müllerian part of the reproductive tract arise from a homogeneous population of epigenotypes which are capable

of following several different functional pathways. Accepting the latter explanation provides a conceptual foundation for the comprehension of hormone-induced pathological changes.

The hypothetical model which will shortly be proposed in this report to account for the manner in which hormonal imbalances can lead to pathology and even neoplasia in the reproductive tract is dependent on an understanding of the possible role of estrogen in the differentiation of its target tissues. A detailed examination of the intricacies of the differentiation process is beyond the limits imposed on this discussion. However a simplified statement of certain concepts is necessary to provide a foundation for later arguments.

The essence of the theory of embryonic differentiation (as understood by the present author) is that in embryonic cells, all of the specialized genotypic instructions (e.g. the parts of the genome coding for the synthesis of the oviductal secretory proteins) are repressed, and it is not until organogenesis occurs that certain 'inducing' substances inhibit those particular 'repressors' which are acting on the parts of the genome which will be utilized in the adult tissue. This process gives rise to a population of epigenotypes or stem-cells which are now specific for that particular tissue. However functional (and morphological) differentiation does not take place until proliferation of the epigenotypes occurs and (presumably) a certain critical mass is reached. At this stage environmental factors inhibit the cells from re-entering the mitotic cycle, proliferation ceases (apart from normal cell turnover) and the cells either

enter a 'resting' state or proceed along a 'functional' pathway. It is when cells enter the 'functional' pathway and begin to perform their characteristic duties that morphological differentiation takes place (e.g. ciliogenesis and secretory granule formation)(see Rutter et al.,1967).

In adult tissues the homeostatic factors which regulate the proliferation and functional differentiation of the cells are not entirely understood. Substances known as 'chalones' (see Bullough,1965) are believed to specifically inhibit mitosis in certain 'target'tissues, and these diffusable substances are considered to be produced by the target cells themselves and/or the neighbouring tissues. Epithelia which have a low turnover rate thus have a high 'chalone' concentration. Mitogenic hormones such as estrogen are believed to cause mitosis in their target tissues by directly inhibiting the chalones(Ibid.).

With a consideration of the above concepts in mind, this author would like to propose the following model concerning the manner in which estrogen influences the differentiation, maintenance and functional derangement of its Müllerian-derived target tissues.

It is proposed that during fetal organogenesis and the development of the Müllerian duct, the coelomic mesothelium lining the Müllerian duct gives rise to a homogeneous population of epigenotypes. At a later stage in fetal development, or during the postnatal period prior to puberty (depending on the species) estrogenic stimulation of the Müllerian duct would provoke proliferation of the epigenotypes and growth of the tissues.

Once a certain amount of proliferation had occurred mitosis would cease and the prevailing hormonal milieu and local tissue factors would dictate to the reproductive tract cells which functional pathway to follow.

Besides stimulating proliferation and influencing the cell's choice of functional pathways, estrogen would also modulate the activity of the adult 'differentiated' cell. This was observed in the present study where a quantitative difference between the amount of estrogen stimulating the differentiated cells of the immature and mature rabbit oviducts led to a quantitative difference in the number and size of the secretory granules found in the oviductal cells.

Of course simply to state that 'estrogen' is responsible for the happenings described above is deceptive. In reality progesterone definitely influences the functional modulations of the adult 'differentiated' cell and there is considerable evidence that the relative amounts of estrogen and progesterone prevailing at the time at which the cell leaves the mitotic cycle play a major part in directing the cell along a particular functional pathway.

For instance, Palmiter and Wrenn (1971) have found evidence suggesting that progesterone given to immature chicks concurrently with estrogen will block the normal differentiation of certain cells which, with solely an estrogenic stimulation, would have developed into tubular gland cells, and instead redirects these cells to a ciliated or goblet cell pathway. The fact that when Arias-Stella (1955) treated ovariectomized rats with a particular

combination of estrogen and progesterone, the uteri of these animals showed an 'oviductal' type of metaplasia (although he did not call it that) whereas with other combinations of these hormones most workers found a stratified squamous metaplasia, also suggests that the prevailing hormonal milieu influences the functional pathway taken by differentiating cells.

At the present time it is difficult to conceive of a mechanism by which the hormonal stimulation reaching the various regions of the epithelium lining the reproductive tract via the blood stream could be sufficiently different (qualitatively and/or quantitatively) to account for the divergent differentiation pathways. Therefore if the concept of a homogeneous population of stem cells is to be valid, the importance of local factors regulating differentiation must be considered.

Several observations support the importance of local factors in the differentiation process. In many species the number of ciliated cells steadily decreases as one progresses from the fimbriated end of the oviduct, which contains predominantly ciliated cells, to the junctura region where ciliated elements are scarce. In Part I of this report the observation of mixed ciliated and secretory cells was discussed in relation to a common origin for the two cell types.

Nevo, Weisman and Sade (1975) have examined the behaviour of several types of muco-ciliary epithelium in tissue culture and they favour the idea of a common epigenotype for the two mature phenotypes. They observed that in monolayer outgrowths developing from tissue explants of rabbit trachea, ciliated cells developed



throughout the monolayer whereas only those cells closest to the tissue explant would differentiate into goblet cells, an observation which stresses the importance of local tissue factors in regulating differentiation.

The involvement of local factors is also suggested when studying heterotopic alterations. The incidence of stratified squamous metaplasia in rodents in response to estrogen-stimulation steadily increases as the vaginal end of the Müllerian duct is approached (where a stratified squamous response to estrogenic stimulation occurs normally during the estrus cycle). Stratified squamous metaplasia rarely occurs in the human oviduct 'normally' (Novak and Woodruff, 1974) or in response to estrogen treatment (this report-rat study). The most common heterotopic alteration in the human oviduct is endometrial metaplasia and the incidence of this change is highest in the isthmus and steadily decreases towards the fimbria (see Novak and Woodruff, 1974,p.297).

The reason that metaplasia is observed so rarely in the oviduct, even following prolonged estrogenic stimulation, may be because of the long life of the oviductal epithelial cells. These cells have the longest turnover time of all of the cells of the rat reproductive tract derived from the Müllerian duct (Bertalanffy and Lau, 1963). Metaplasia occurs in a tissue when cells leaving the mitotic cycle are directed along a functional pathway that is considered 'foreign' to that tissue. Therefore the higher the cell production rate the greater the opportunity is for metaplasia to occur.

### Exogenous Estrogen and Pathology in the Reproductive Tract

The results of this study have suggested several ways in which the prolonged administration of estrogen can lead to pathology in the female reproductive tract. The initial effect of estrogen pellet implantation was to upset the hypothalamico-pituitary-ovarian axis. The resulting alteration in the hormonal stimulation (exogenous and endogenous) of the target tissues caused several things to occur. One was the initiation of changes in the reproductive tract which facilitated the establishment and spread of bacterial (and possibly viral) infections.

A change in the relative levels of estrogen and progesterone also affected the target cells directly. The hormonal imbalance disturbed the functioning of the rat nonciliated oviductal cells in such a manner as to prevent proper formation and release of secretion granules, thus leading to the development of intracellular mucus inclusions (microvillous vesicles). In the rabbit, the continued estrogenic stimulation led to an overabundance of secretion production, which in the absence of progesterone, could not be released and which in some instances appeared to cause breakdown of the basal cell membranes and leakage of the secretory product into the intercellular space and lamina propria.

The upset in estrogen/progesterone ratio also led to metaplastic change in the reproductive tract. Stem cells leaving the mitotic cycle to enter a functional pathway appear to have been provoked by the altered hormonal milieu to enter a different pathway. That all of the metaplastic alterations seen in the

estrogen-treated rats were typical of epithelium elsewhere in the reproductive tract was taken as evidence in favour of a common stem cell type for all of the reproductive tract epithelia derived from the Müllerian duct.

Prolonged estrogenic stimulation also led to hyperplasia and adenomyosis in the rabbit oviductal epithelium, and in one instance, to neoplasia. Once endogenous progesterone secretion had ceased in the estrogen-treated rats, the unopposed action of estrogen led to the development of foci of oviductal epithelial 'proliferation'. These 'proliferative' nodules may have represented premalignant lesions of the oviductal epithelium.

In addition to stimulating the 'normal' reproductive epithelia to abnormal proliferation, the prolonged estrogen treatment also stimulated the metaplastic epithelium. For example, serosal mesothelium which had 'differentiated' into oviductal epithelium also developed proliferative nodules. And the morphology of the one case of uterine neoplasia observed in an estrogen-treated rat in the present study suggested an abnormal proliferation of uterine cells demonstrating metaplasia to oviductal elements (containing mucus inclusions).

This observation suggested a hypothetical mechanism by which estrogen could be directly responsible for the development of certain cancers. The presence of tissue-specific chalones and the manner in which these substances inhibit mitosis in their target tissues has already been mentioned. It has also been stated that mitogenic hormones such as estrogen act by opposing chalones.

This author proposes that an upset in the normal hormonal milieu, possibly initiated by exogenous estrogen or an upset

in endogenous hormone production (e.g. the development of an estrogen-secreting tumour), initially leads to metaplastic change in an epithelium. Continued estrogenic stimulation may then cause abnormal proliferation of the metaplastic cells. The mitogenic effect of the estrogen would be stronger than usual because the chalone produced by the adjacent tissues would not have an effect on the metaplastic cells because they would be specific for the original, rather than the metaplastic, tissue.

The above suggestion is only hypothetical, but there is some evidence which is compatible with this explanation. As already mentioned, the one uterine neoplasm found in this study appeared to have developed from metaplastic uterine epithelium. In their study, Meissner, Sommers and Sherman(1957) stated that "metaplasia in the adjacent endometrium was a consistent and striking accompaniment of all the cancers". In addition, two of the six cancers which developed in their stilbesterol-treated rabbits were adenoacanthomas, which might have arisen partly from stratified squamous metaplastic epithelium.

Enough cases of adenoacanthoma of the ovary (which seems to indicate an endometrial origin) have been reported to suggest to several authors that endometriosis might be regarded as a premalignant disease (see Novak and Woodruff,1974). Anbrokh(1970) describes cases of adenocarcinoma of the oviduct which have developed from foci of endometriosis, but says that squamous carcinoma developing from metaplastic epithelium is extremely rare in this organ. This is consistent with the infrequent occurrence of this type of metaplasia in the oviduct.

And finally Reintoft, Lange and Skipper (1974) reported a case of endometrial carcinoma of the rectum in a patient who also had a granulosa cell tumour of the ovary. These authors also cited a report of a patient with another type of ovarian estrogen-secreting tumour (i.e. a thecoma) who developed an adenocarcinoma in an umbilical focus of endometriosis.

A similar situation may be behind the development of vaginal adenocarcinomas in young women as a result of maternal stilbesterol treatment during pregnancy (see Introduction, pp.27-29). Forsberg (1972) has postulated that these cancers arise from 'untransformed' Müllerian epithelium which has persisted in the vagina because of an upset in differentiation caused by the administration of stilbesterol (to the mother) during a critical period of fetal reproductive tract development. Endogenous estrogen production at puberty stimulates these cells to proliferate and form adenomatous growths with subsequent malignant change. In the human vagina (c.f. other species) none of the 'adult' cells are considered to have been derived from Müllerian epithelium (Ibid.).

Therefore it is quite likely that the chalones produced by the subepithelial vaginal tissues, and even by the surrounding normal vaginal epithelium will have no effect on the 'Müllerian' cells and will be totally incapable of counteracting the proliferative changes instigated by the pubertal estrogen secretion.

#### Final Comment

Many of the findings cited in this discussion suggest the importance of the interplay between local tissue factors and the

immediate hormonal milieu in determining the future for any particular cell. This interplay has also been discussed in relation to a hypothetical mechanism by which estrogen can influence the differentiation of its target tissues, the regulation of the function of the differentiated cells, and the behavioural derangement of target cells resulting in pathological changes.

Unfortunately scientific technology has not reached the stage where the stimuli reaching an individual cell can be monitored directly. It was a traumatic experience for this author to realize that the interpretation of the results of the present study hinged on an understanding of how the exogenous estrogen was affecting the whole animal, rather than what it might be doing to the oviduct directly. And during the course of this study it has become even more apparant how fragile the basis on which certain conclusions are built.

The paradoxical findings of the many experiments examining the effects of exogenous estrogen on the intact animal (Arias-Stella, 1955 c.f. this report and others) illustrate how even subtle differences in, for example, the levels of estrogen and progesterone circulating in the blood, can alter the results. The conclusions drawn in this report have been based on the morphological findings of the present study, and the interpretation of these results in the light of previously reported experimental physiological and biochemical data has been tempered with the knowledge that not all of the findings of similar previous studies may be applicable.



FIGURES 43-72

Figure 43: Average weights of pituitary, ovaries (2), oviducts(2) and uterus, and average body weight of control rats and experimental rats following 1-4 months of estrogen treatment.

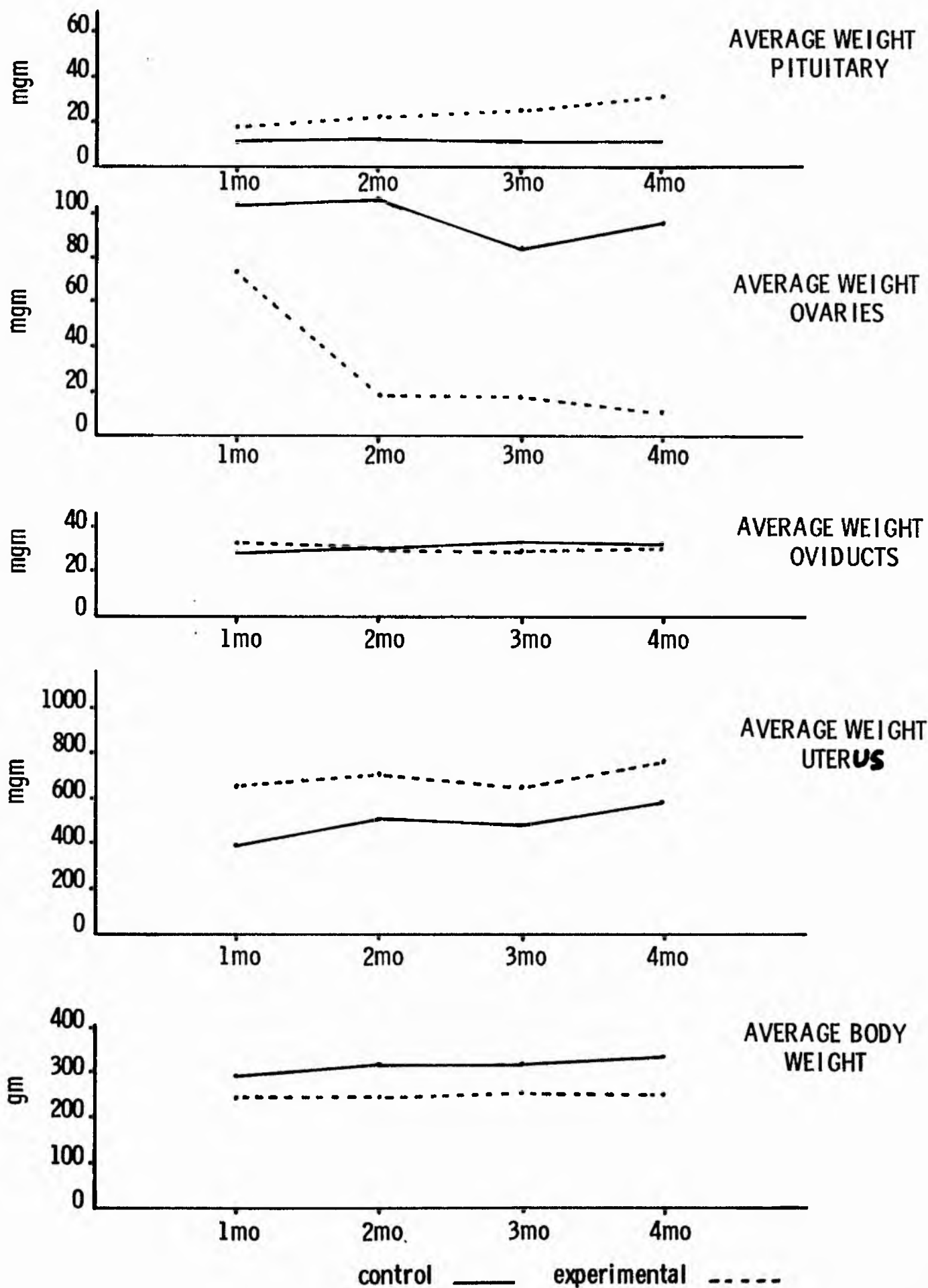


Figure 44: Rat ovary.

a. Control. H&E. X16

CL- corpus luteum

b. Following one month of estrogen treatment. H&E. X16

CL- corpus luteum

c. Following four months of estrogen treatment. H&E. X16

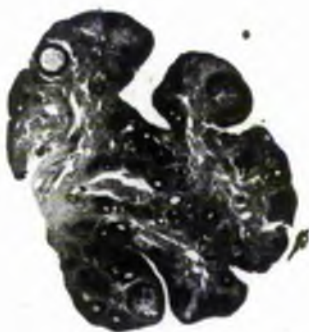
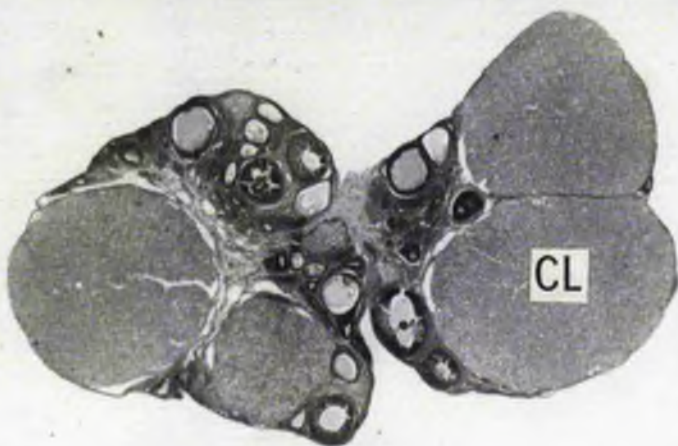
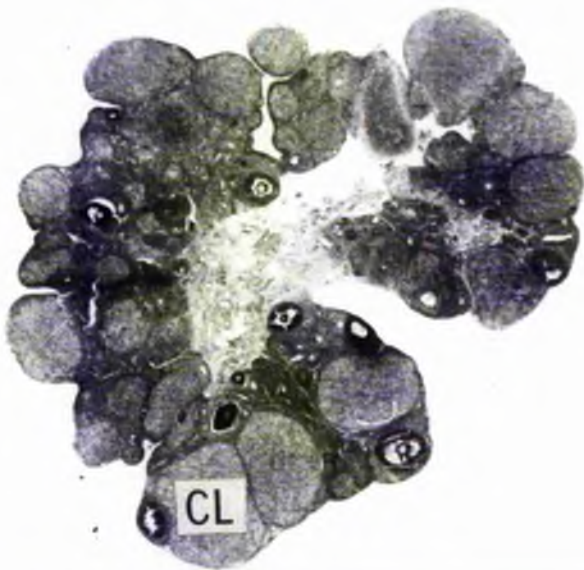


Figure 45: Rat uterus. Ninety days estrogen. Alcian Blue. X400

SE- surface epithelium

G- glandular epithelium

→ - Alcian Blue +ve inclusions

Figure 46: Neoplasm of rat uterus. Two-hundred and twelve days  
estrogen. Alcian Blue.

a. X64 M- myometrium

b. X160 Arrows indicate Alcian Blue +ve inclusions



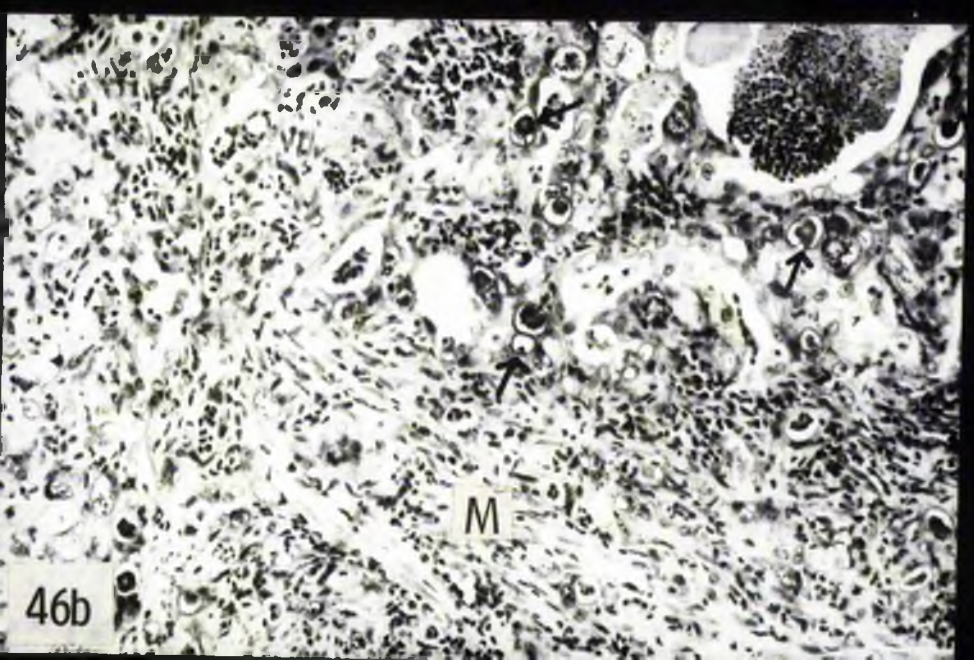
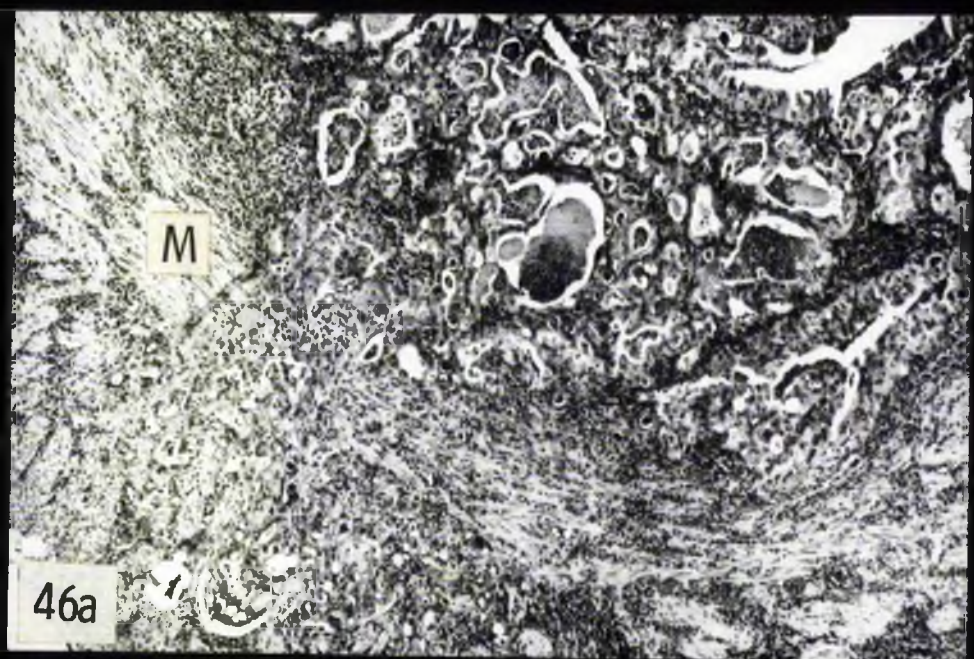


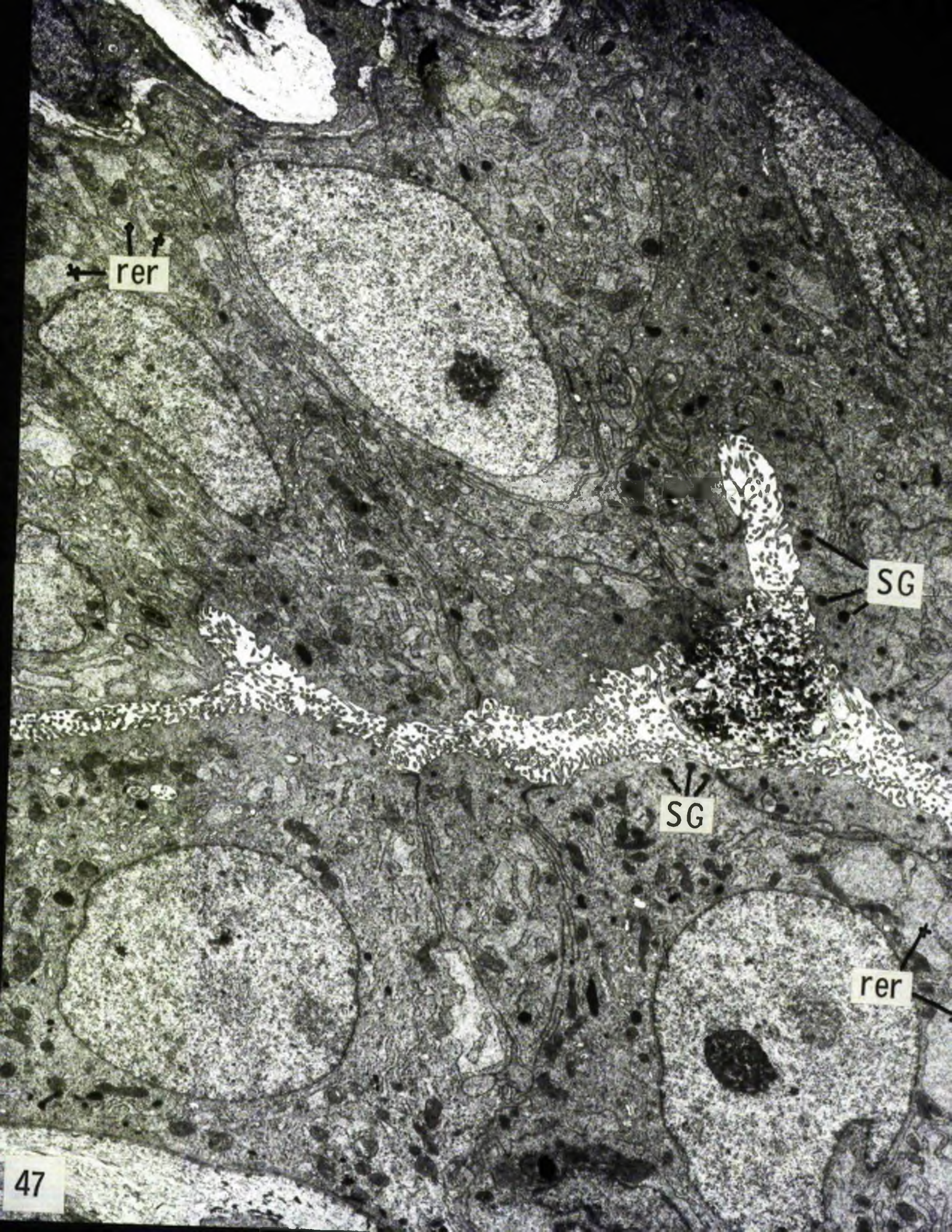
Figure 47: Rat oviduct; isthmus. One hundred and five days

estrogen. UA&LC. X6510

SG- secretory granules

rer- rough endoplasmic reticulum





rer

SG

SG

rer



Figure 48: Rat oviduct; isthmus. One hundred and twelve days  
estrogen. Toluidine Blue. X400

Note flattened mucosal folds.

Figure 49: Rat oviduct; junctura. Sixty days estrogen. Alcian  
Blue. X160

Note abundant Alcian Blue +ve inclusions (→).

Figure 50: Rat oviduct; isthmus. Formation of intracellular  
microvillous vesicles. One hundred and five days estrogen. UA&LC. X23,670

C1- cell showing distended segments of rough endoplasmic  
reticulum

C2- fusion of distended segments of endoplasmic retic-  
ulum with large vesicle. Note that microvilli have  
not yet formed in the vesicle.

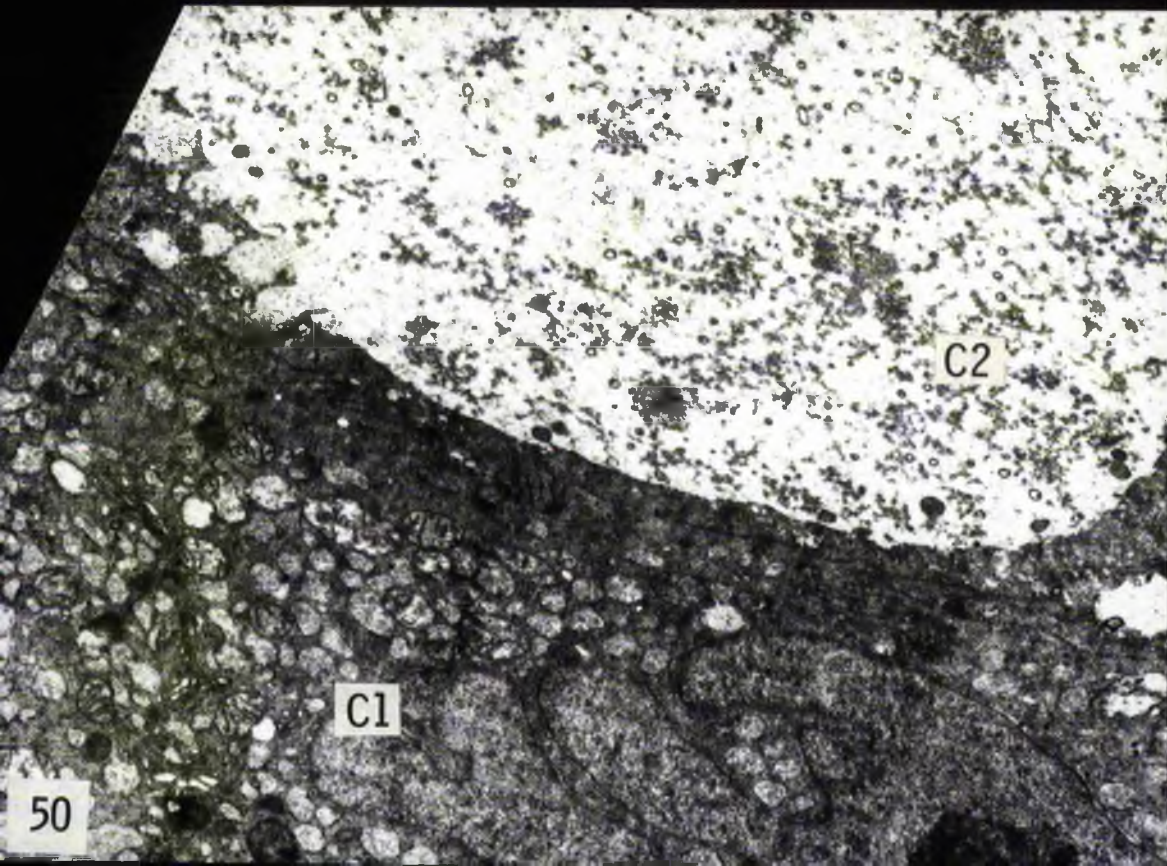
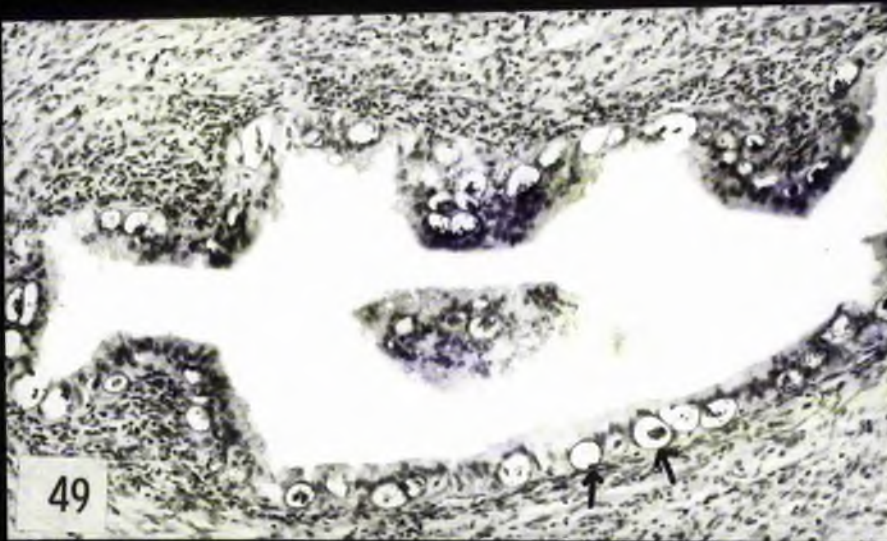
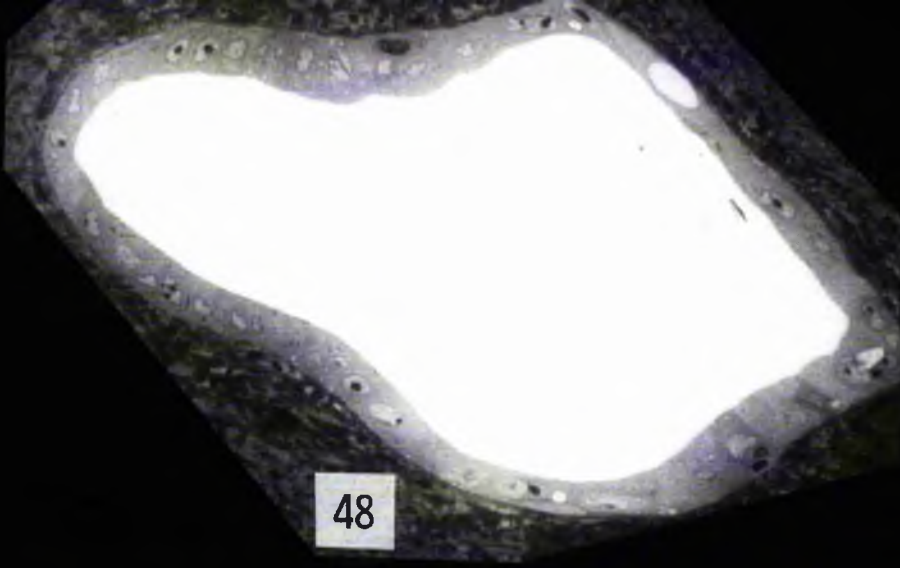


Figure 51: Rat oviduct; isthmus. One hundred and five days estrogen. UA&LC. X6510

Note distended segments of rough endoplasmic reticulum (rer) in adjacent cells.

MV- microvillous vesicle





rer

MV



Figure 52: Epithelium from a rat oviduct with an acute inflammation of the mucosa.

Two hundred and ten days estrogen. UA&LC. X4185

LP- lamina propria

MV- microvillous vesicle

P- polymorphs

→ - duplication of basal lamina



Figure 53: Epithelium from a rat oviduct with an acute inflamma-

tion of the mucosa showing polymorphs and macrophages  
trapped in an 'intraepithelial abscess'. Onehundred  
and sixty-seven days estrogen. Toluidine Blue. X800

LP- lamina propria

M- macrophage?

P- polymorphs

→ - polymorph passing through the epithelium

Figure 54: Rat oviductal isthmic epithelial cells containing

what are believed to be giantleukocytophagic lysosomes(GLL).

Eighty-two days estrogen. UA&LC. X6510



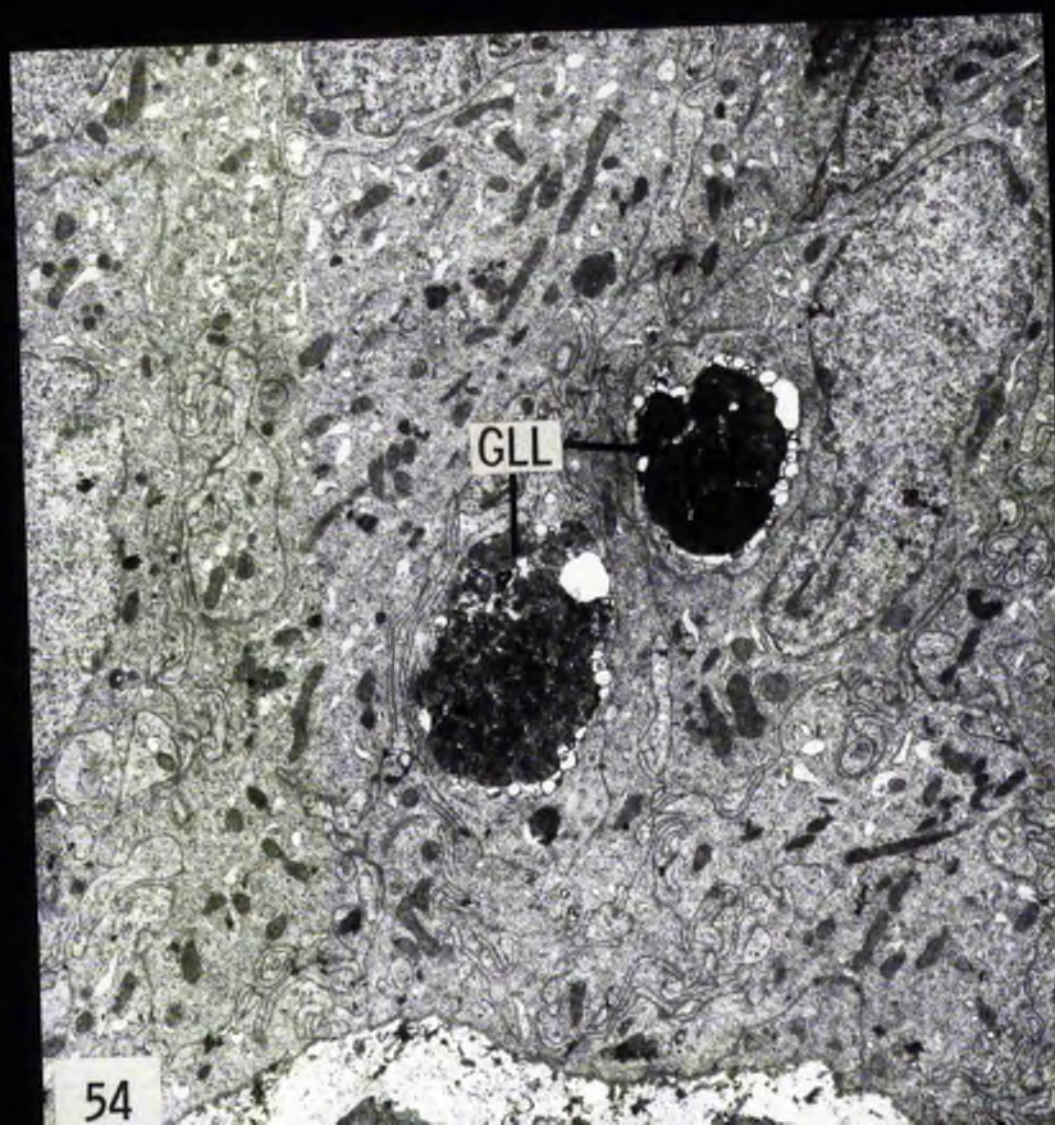
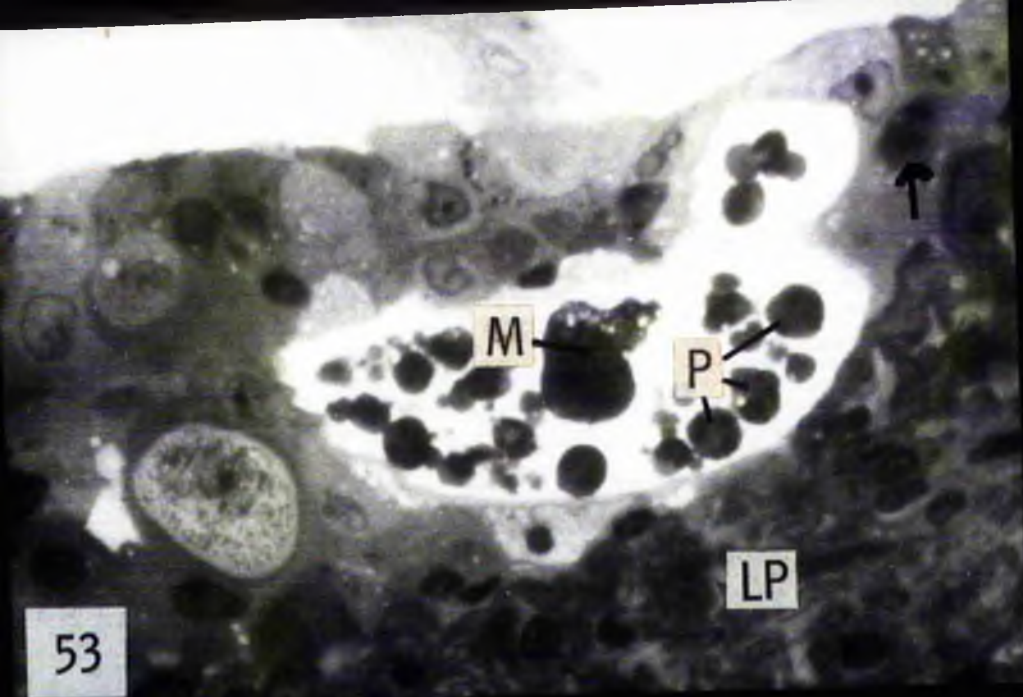


Figure 55: Acute inflammation of the rat oviductal mucosa showing duplication of the epithelial basal lamina(BL). Two hundred and ten days estrogen. UA&LC. X22,500

Figure 56: Acute inflammation of the rat oviductal mucosa showing stratified squamous metaplasia of the epithelium. One hundred and ten days estrogen. H&E.

a. X160

b. X400 Note the acinus of pale cells underlying the stratified squamous epithelium.



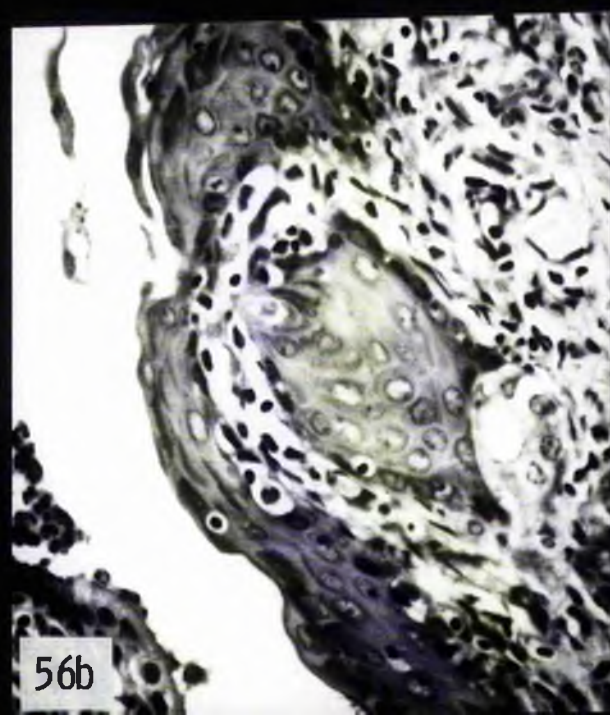
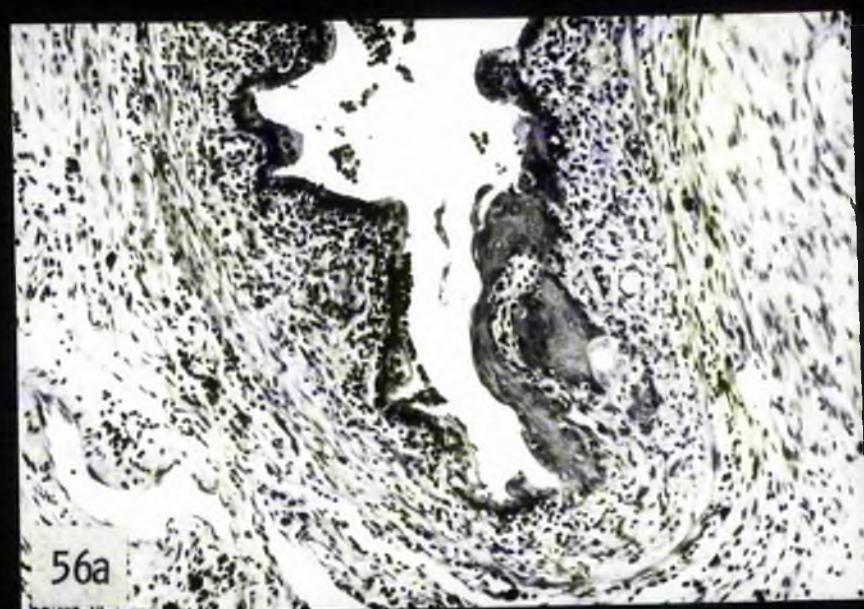
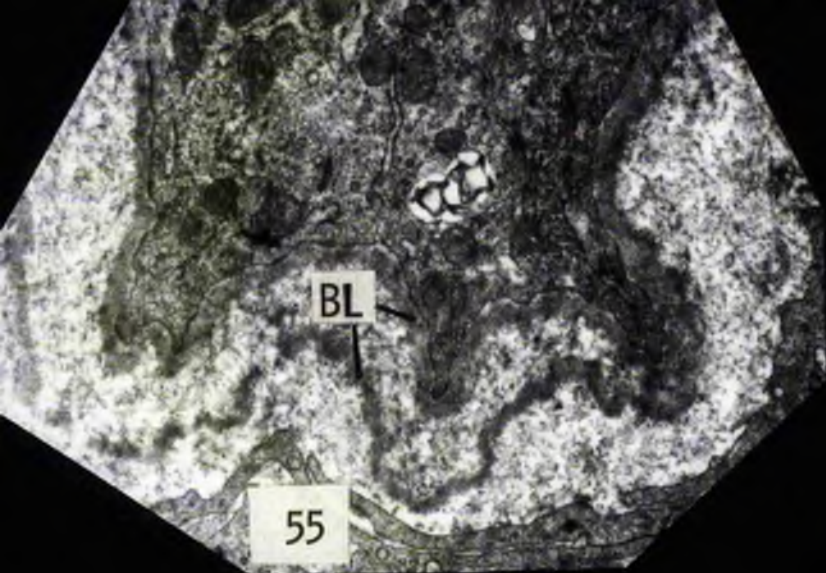


Figure 57: Rat oviduct; hydrosalpinx. One hundred and forty-one days estrogen. H&E. X160

Figure 58: 'Proliferative' configurations of the rat oviductal epithelium.

a. One hundred and twenty days estrogen. H&E. X160

b. One hundred and twenty days estrogen. H&E. X400



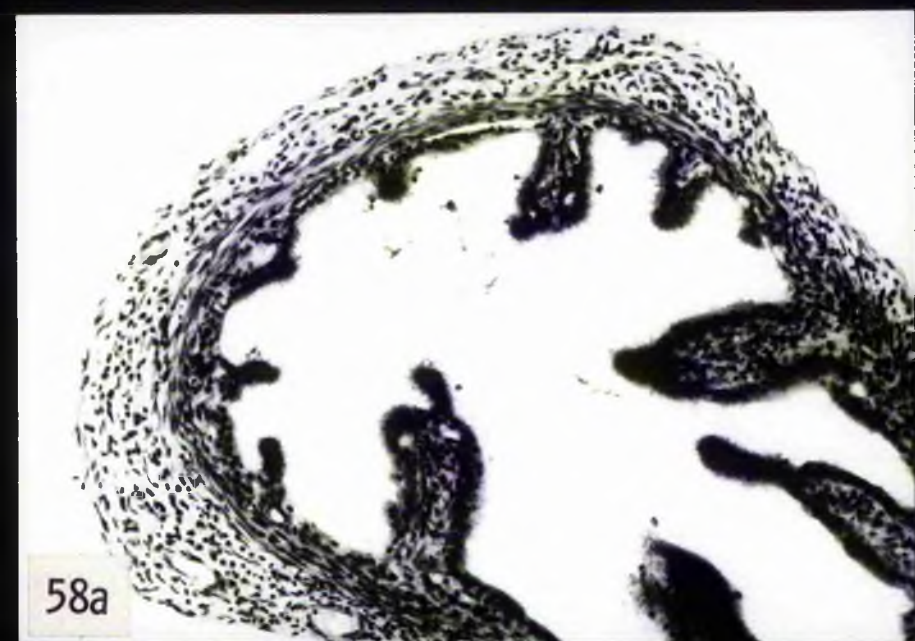
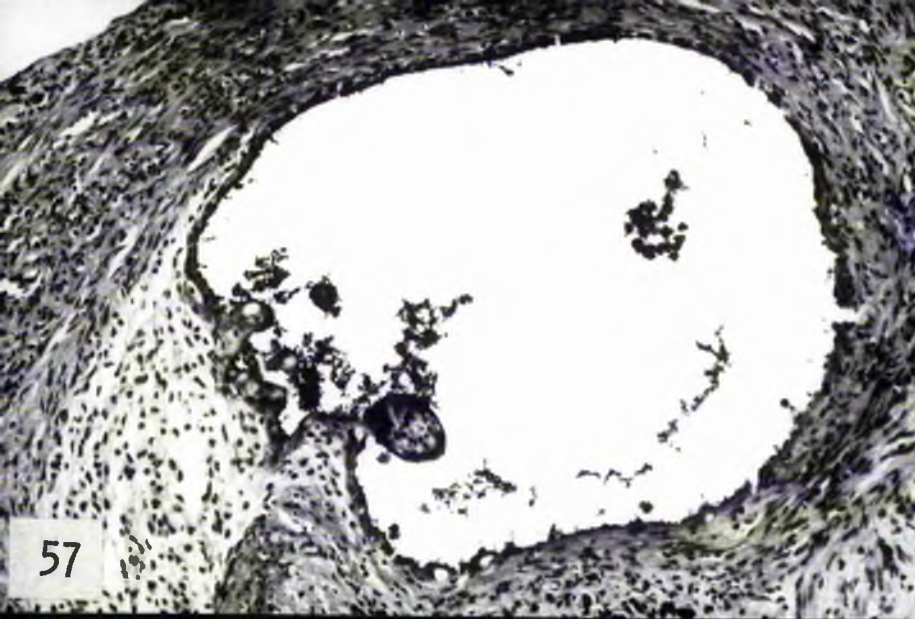


Figure 58 (continued):

c. Eighty-two days estrogen. Toluidine Blue. X1600

d. One hundred and twelve days estrogen. Toluidine  
Blue. X400

e. One hundred and forty-one days estrogen. H&E. X400  
Note underlying acinus of pale cells at arrow.

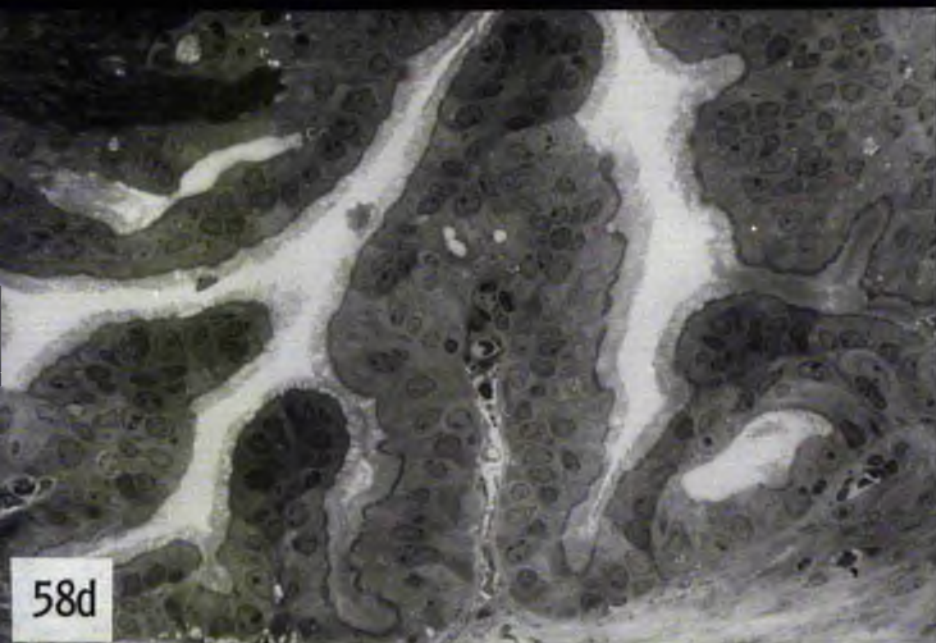
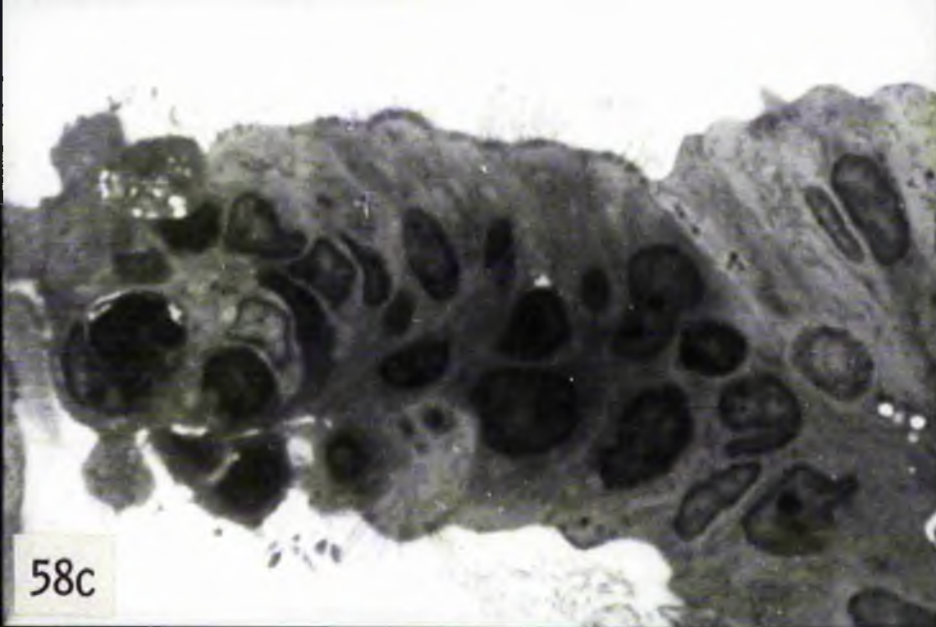


Figure 59: Rat oviduct. One hundred and forty-one days estrogen.

a. Bursal mesothelial proliferation (BP), invagination of oviductal serosal mesothelium with 'metaplasia' to uterine glandular epithelium(→ ) and hydrosalpinx(H). H&E. X64

b. Section of same oviduct as in 'a' showing details of mesothelial invagination with uterine metaplasia. H&E. X160

c. Another section of the hydrosalpinx seen in 'a' showing unusual stratified metaplasia of the oviductal epithelium. H&E. X160



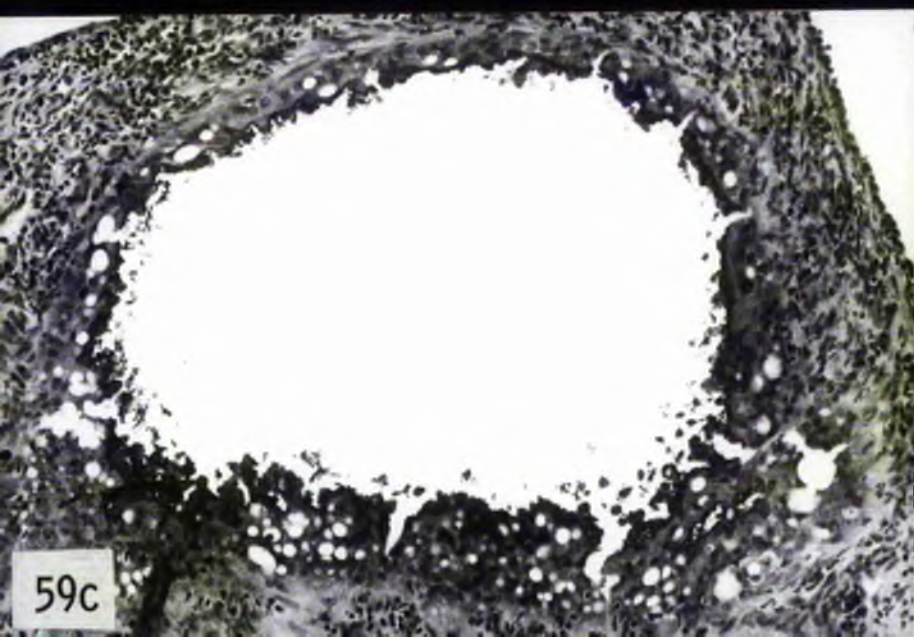
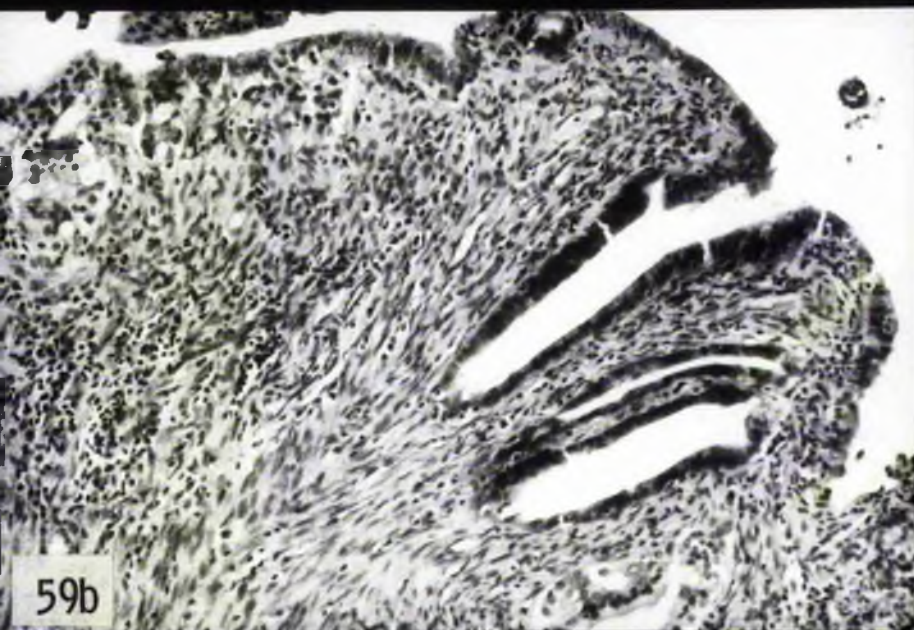


Figure 60: Rat oviduct exhibiting adenomyosis and a chronic  
perisalpingitis. One hundred and forty-one days estrogen.  
H&E. X160

Figure 61: Reaction of oviductal serosal mesothelium to long-  
term estrogen treatment.

- a. Mesothelial inclusion showing oviductal metaplasia.  
Ninety days estrogen. H&E. X160



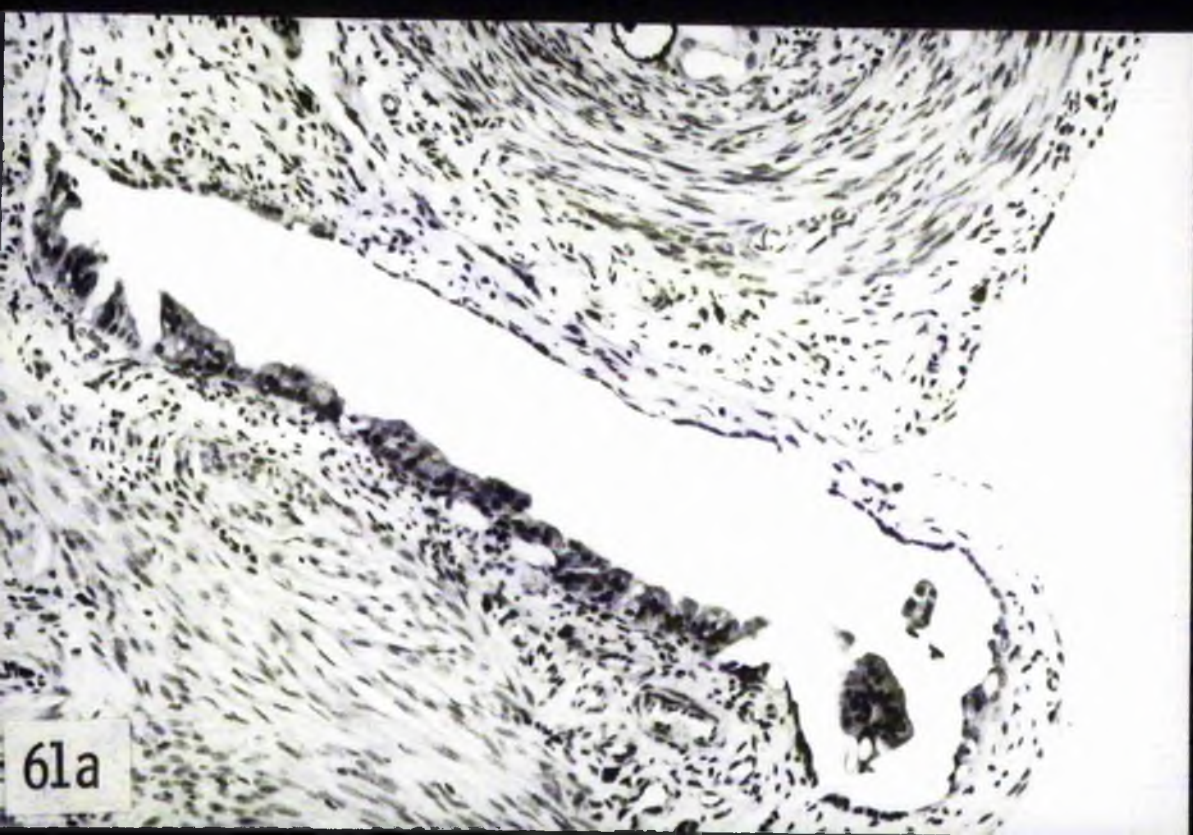
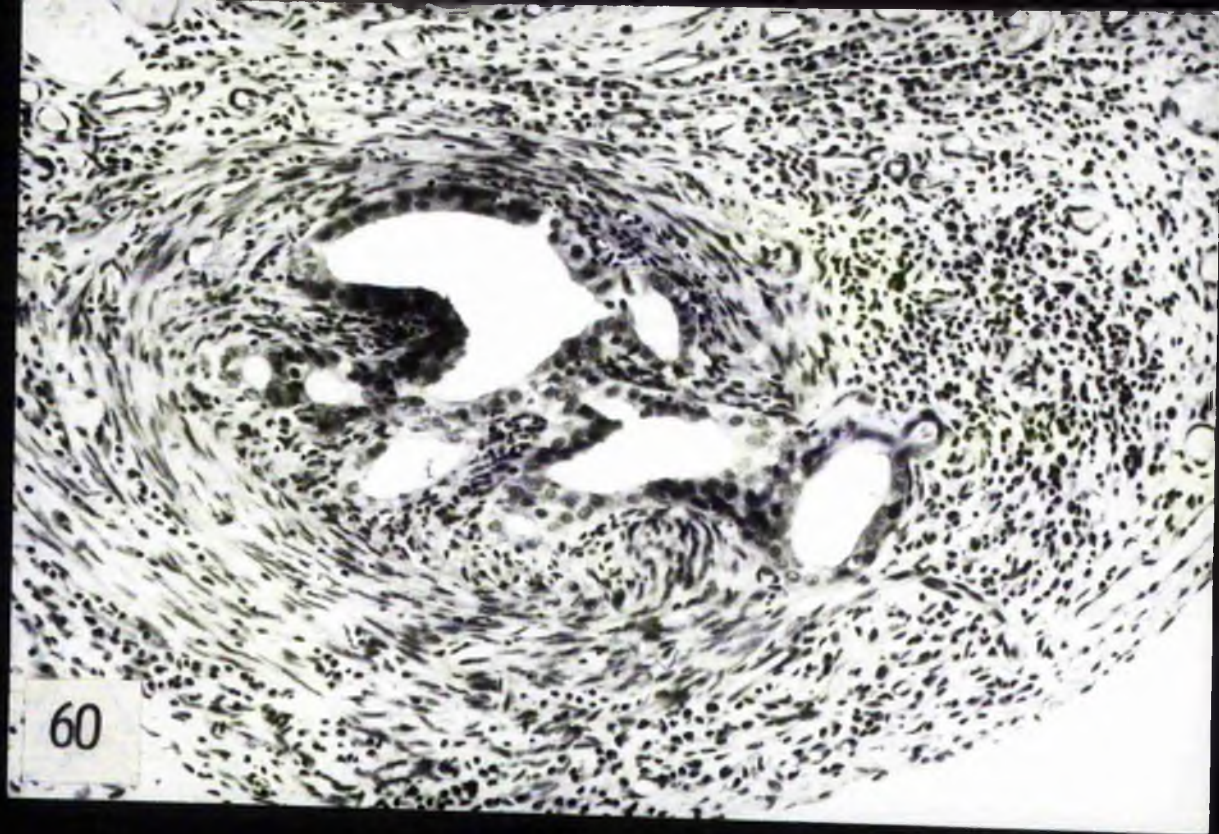


Figure 61 (continued):

b. Mesothelial inclusion showing oviductal metaplasia in some areas and simulating adenomyosis. Sixty days estrogen. H&E. X160

c. 'Metaplastic' surface mesothelium exhibiting 'proliferations' similar to those seen in the oviductal epithelium. Ninety days estrogen. H&E. X400

d. Proliferation of oviductal serosal mesothelium. Ninety days estrogen. H&E. X160



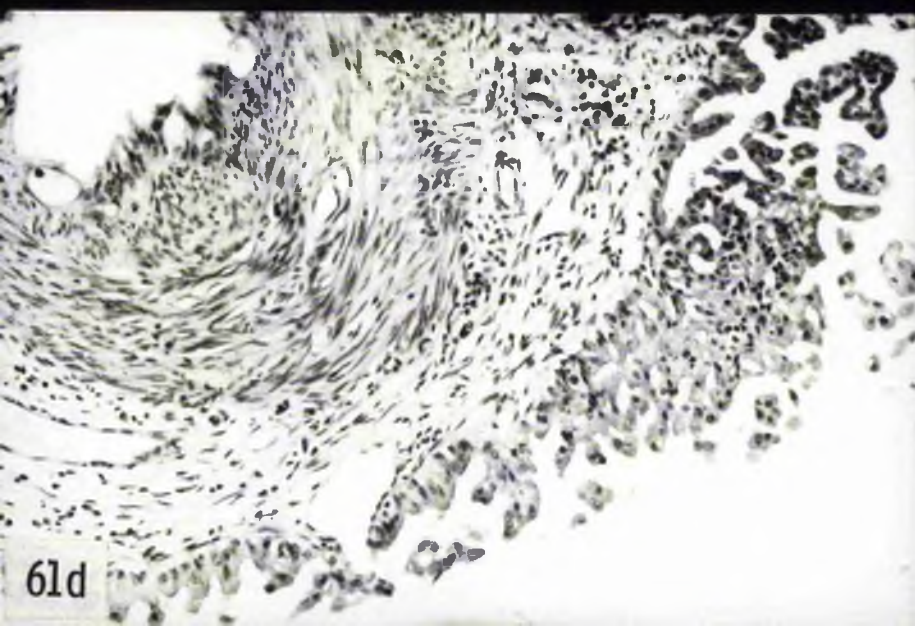
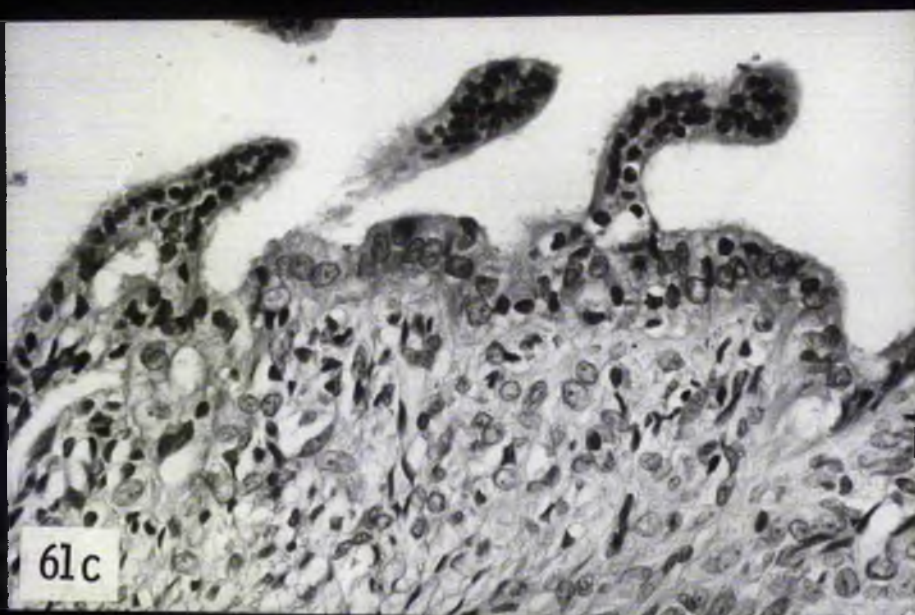
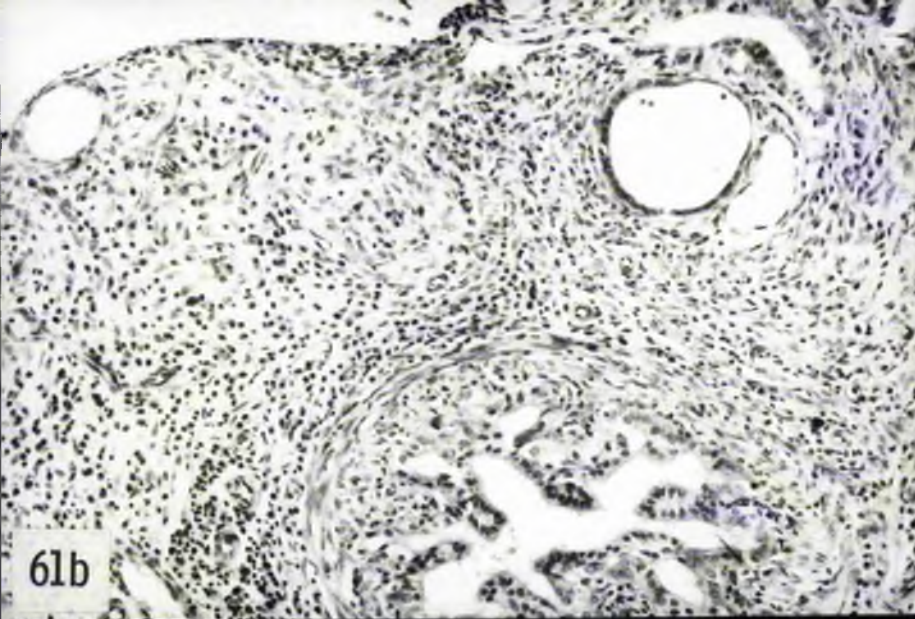
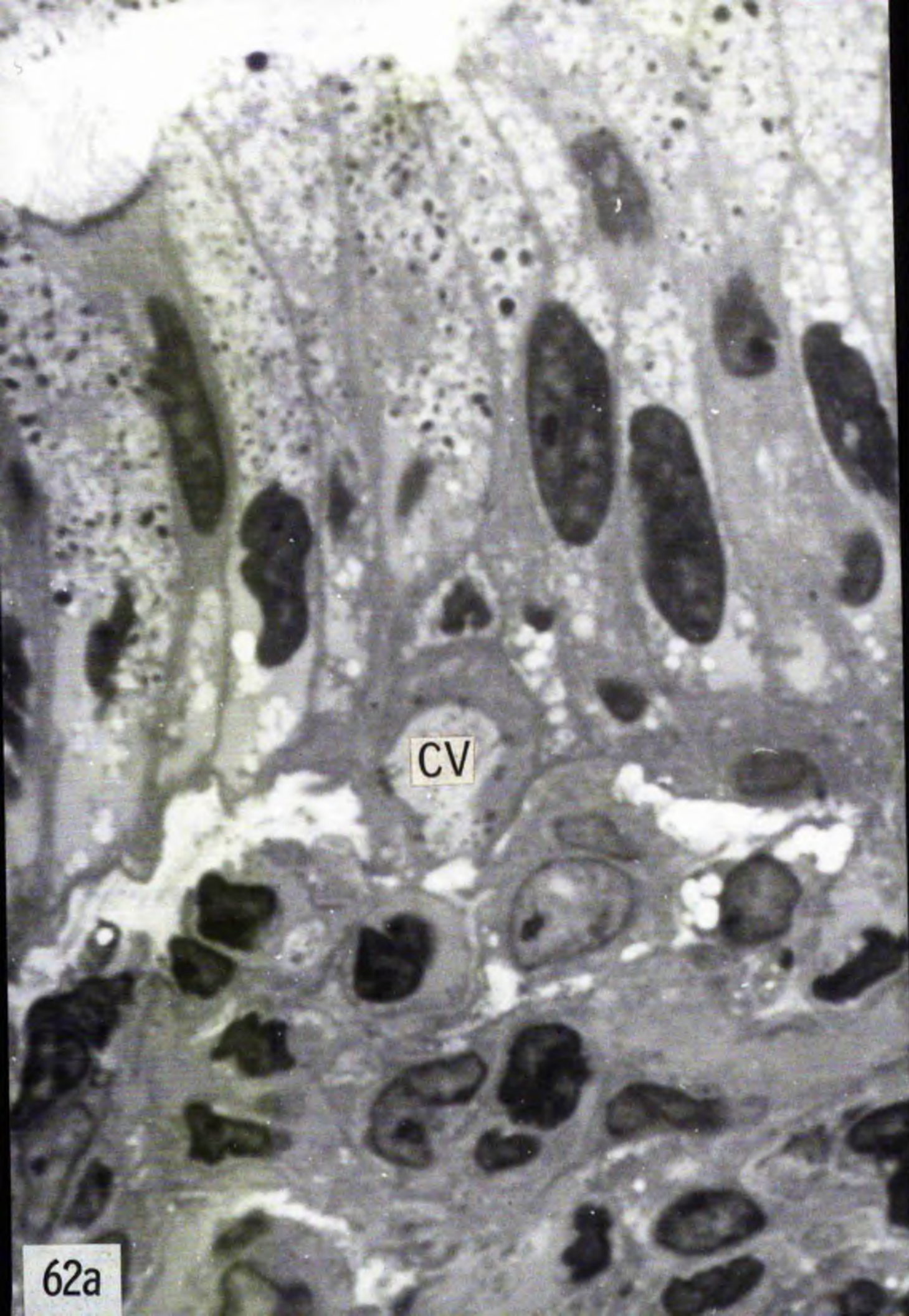


Figure 62: Rabbit oviduct. One hundred and fifty-three days  
estrogen.

a. Toluidine Blue. X2000

CV- ciliary vacuole





62a

Figure 62 (continued):

b. Apices of cells from oviductal epithelium shown in 'a'.

UA&LC. X6510

Note cell with degenerating cilia and viral particles (→ ).

c. Bases of same cells. UA&LC. X6510

rer- enormously dilated cisterna of rough endoplasmic  
reticulum

BL- basal lamina



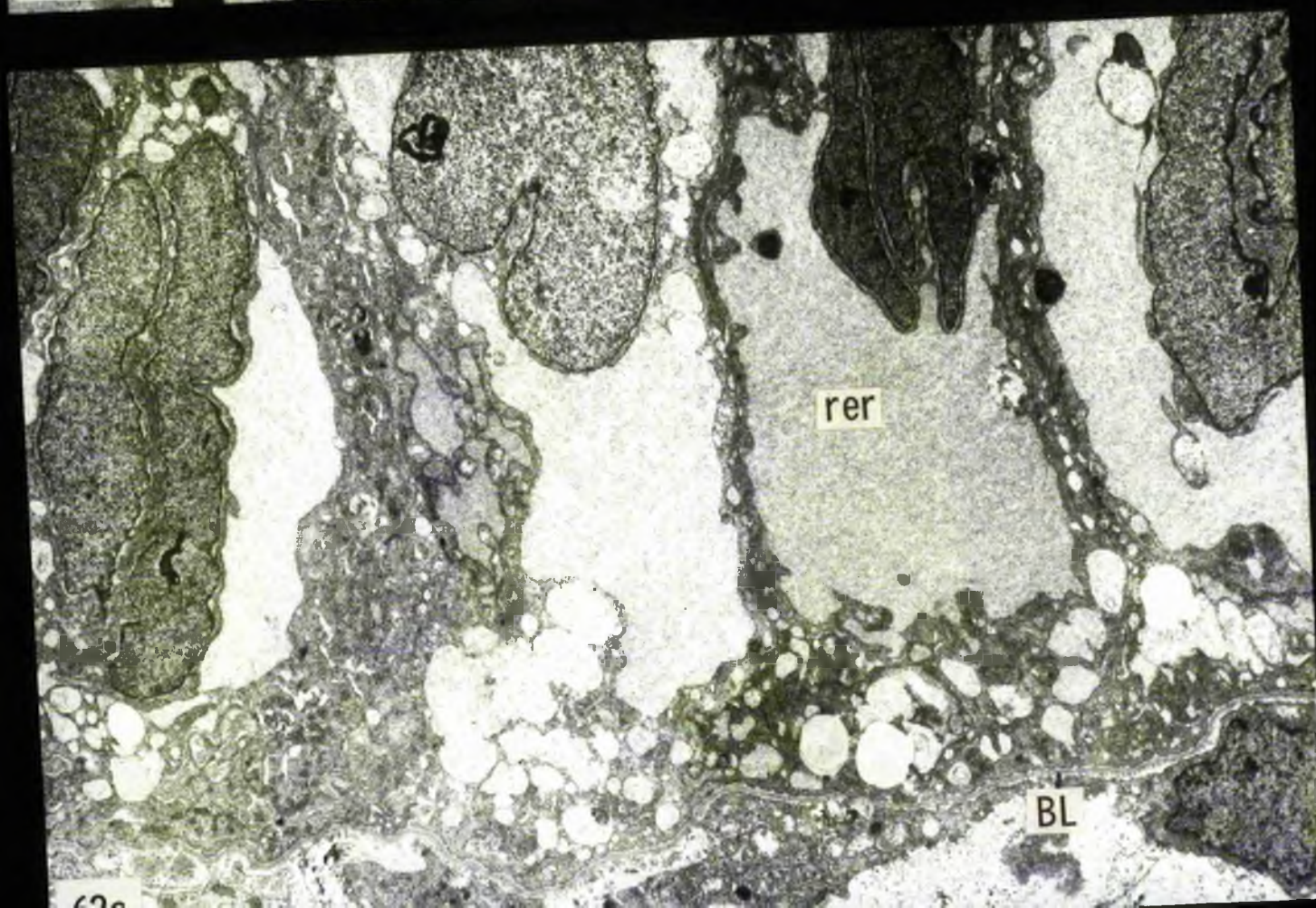
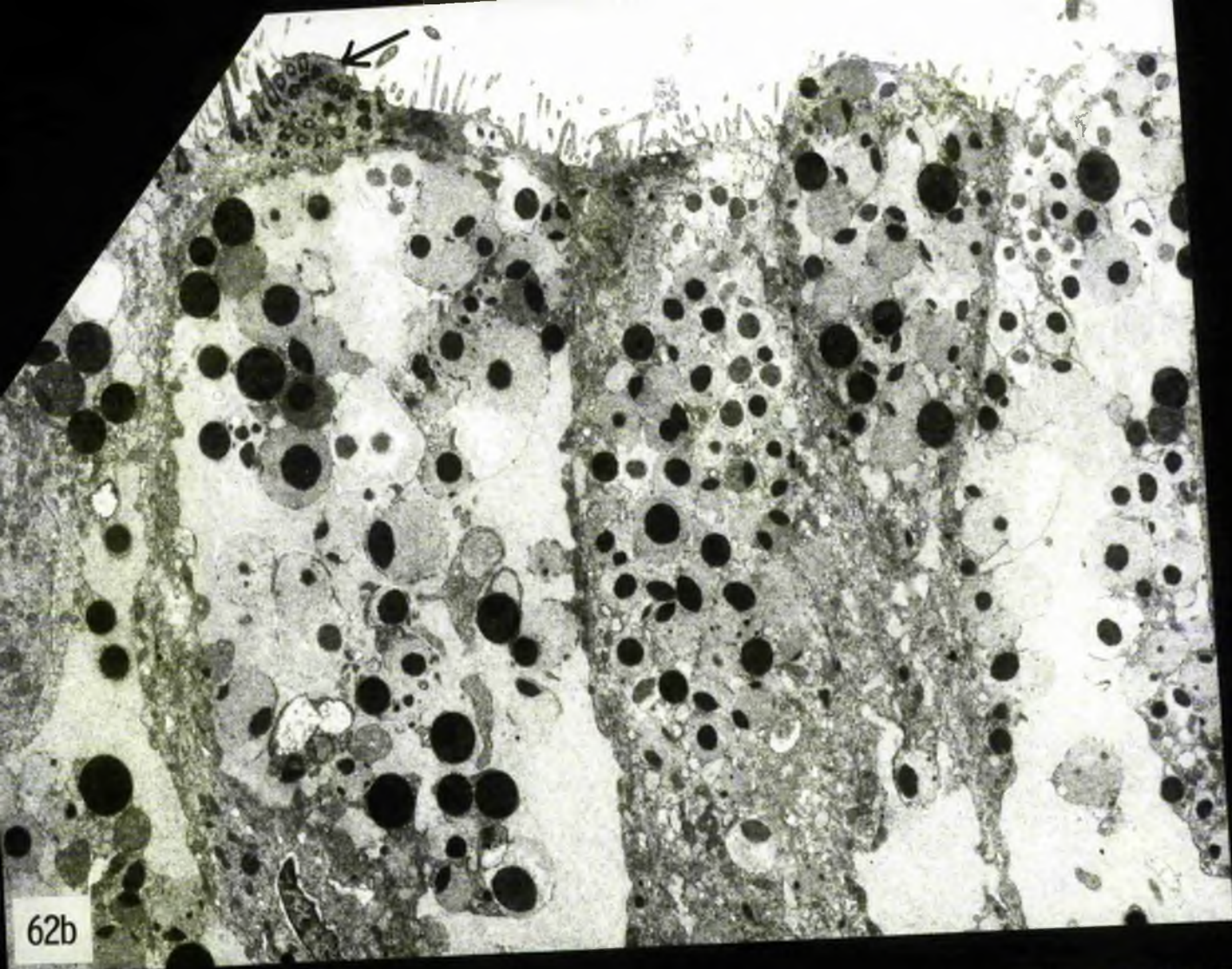




Figure 63: Rabbit oviduct. Three hundred and fifty-five days  
estrogen. UA&LC. X5580. Arrow indicates gap in basal  
lamina through which flocculent material has apparently  
leaked from the epithelium to the lamina propria.  
s- 'lakes' of secretion

Figure 64: Rabbit oviductal epithelium showing nuclear crowding,  
cellular tufting and trapped mucus. Two hundred and  
ninety-four days estrogen. H&E. X160

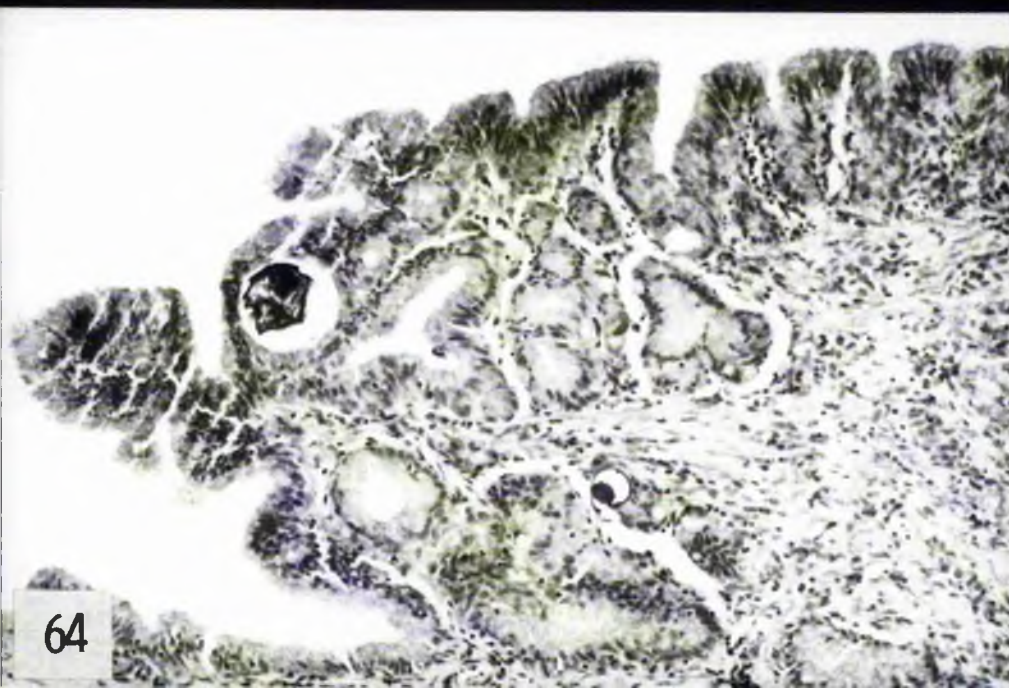
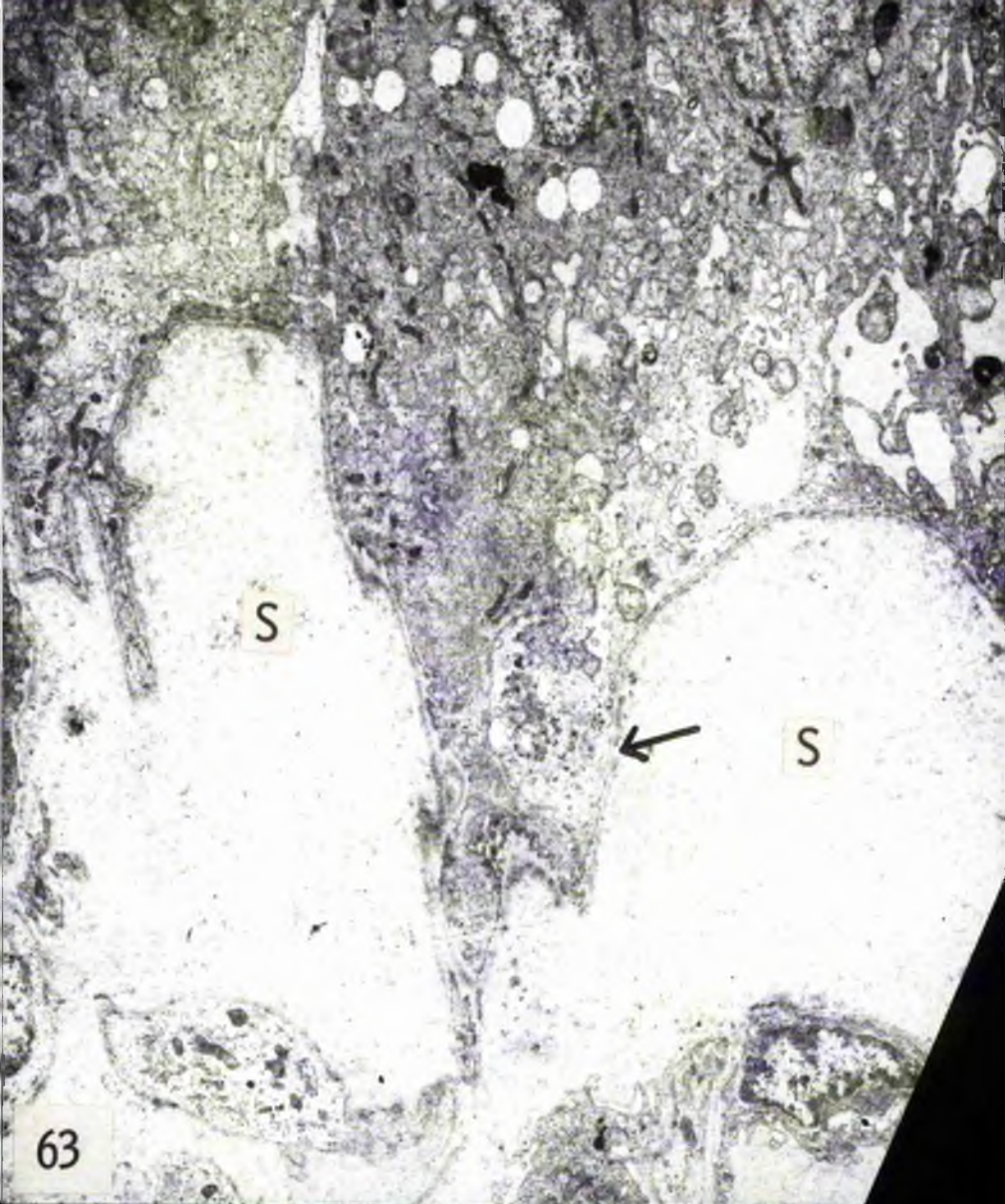
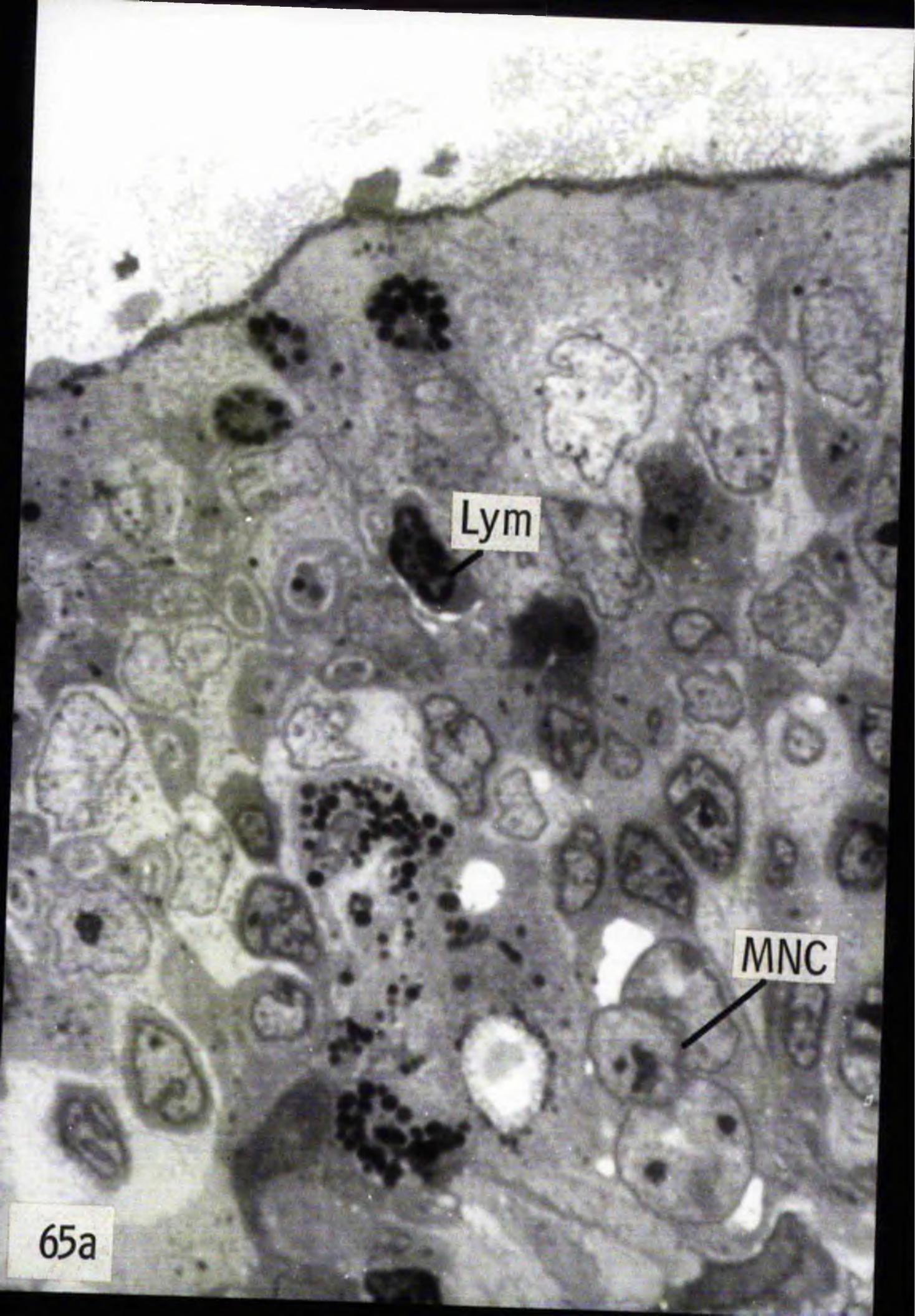


Figure 65: Inflamed rabbit oviduct showing areas of epithelial stratification and hyperplasia. Two hundred and ninety-four days estrogen.

a. Toluidine Blue. X2000 Note what appears to be a multinucleated cell (MNC).

Lym-lymphocyte





Lym

MNC

65a

Figure 65 (continued):

- b. Multilayered epithelium underlying what appears to be typical columnar oviductal epithelium. Toluidine Blue. X800 Note cytoplasmic inclusion (I).  
BL-. basal lamina

- c. Epithelium with cellular inclusions (I) and intra-epithelial lymphocytes (Lym). Toluidine Blue. X800



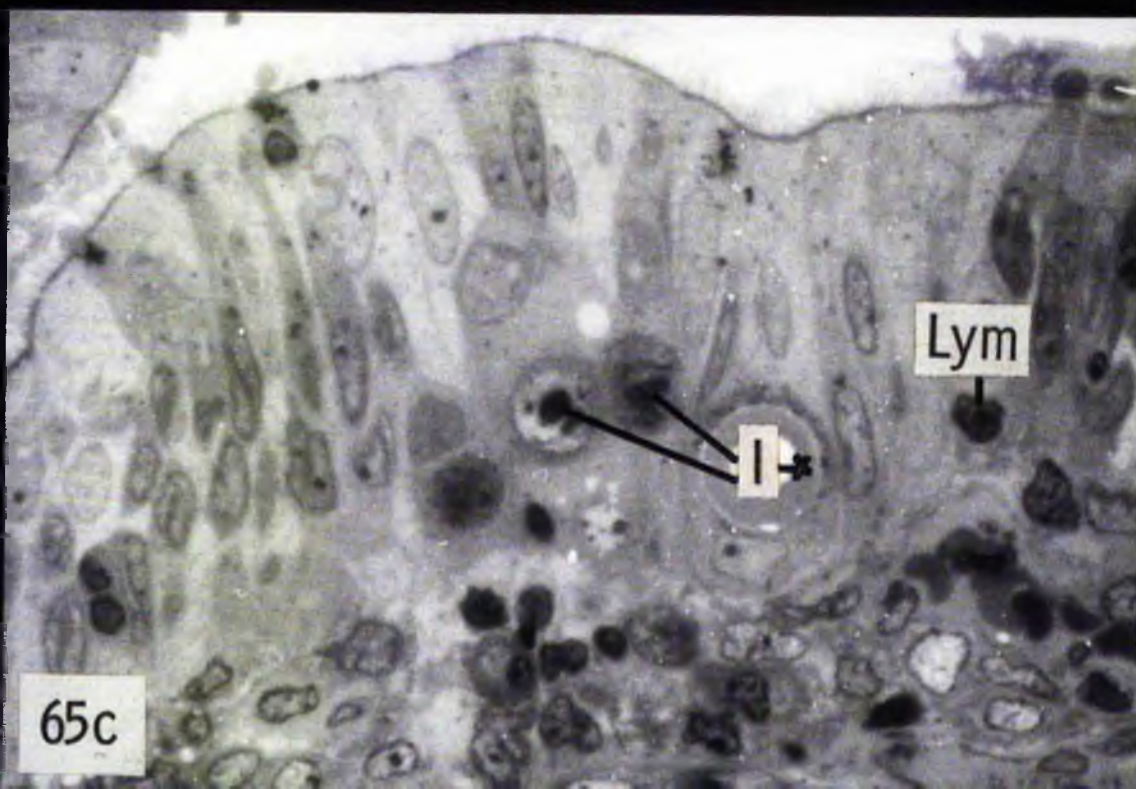
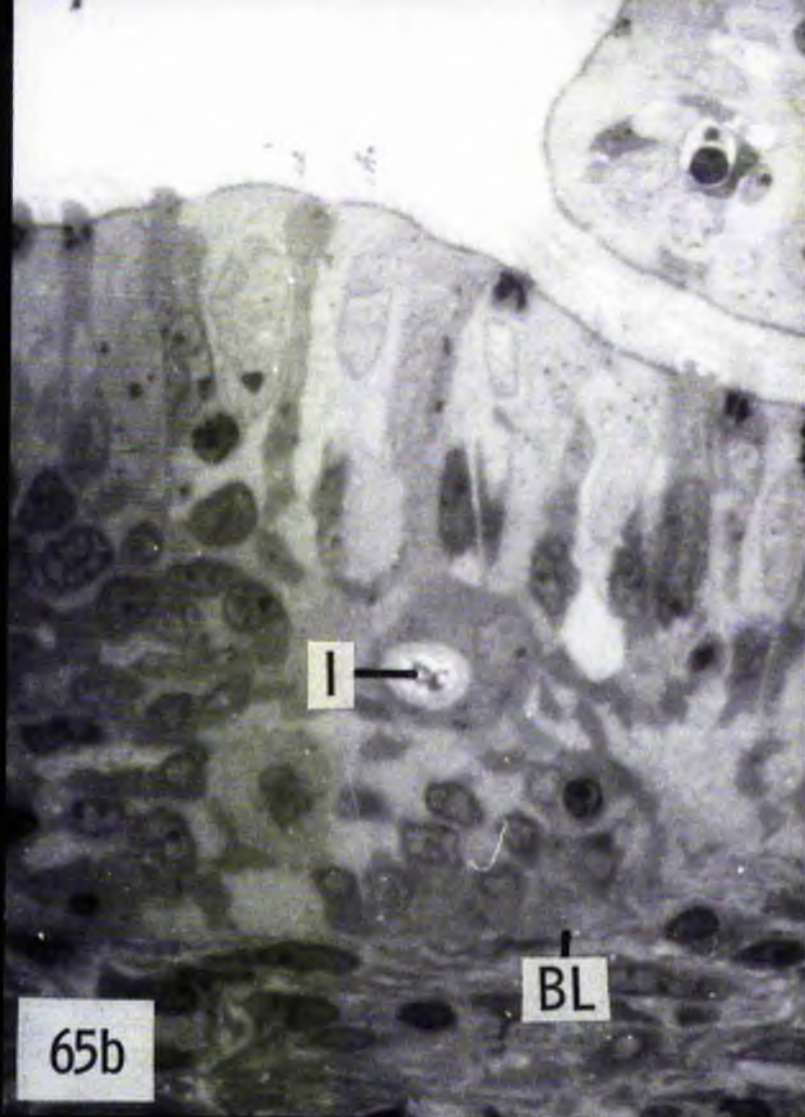


Figure 66: Rabbit oviduct showing lymphocytic infiltration of the epithelium.

Two hundred and ninety-four days estrogen.

a. Electron micrograph of area of epithelium similar to that seen  
in 65b. UA&LC. X3710

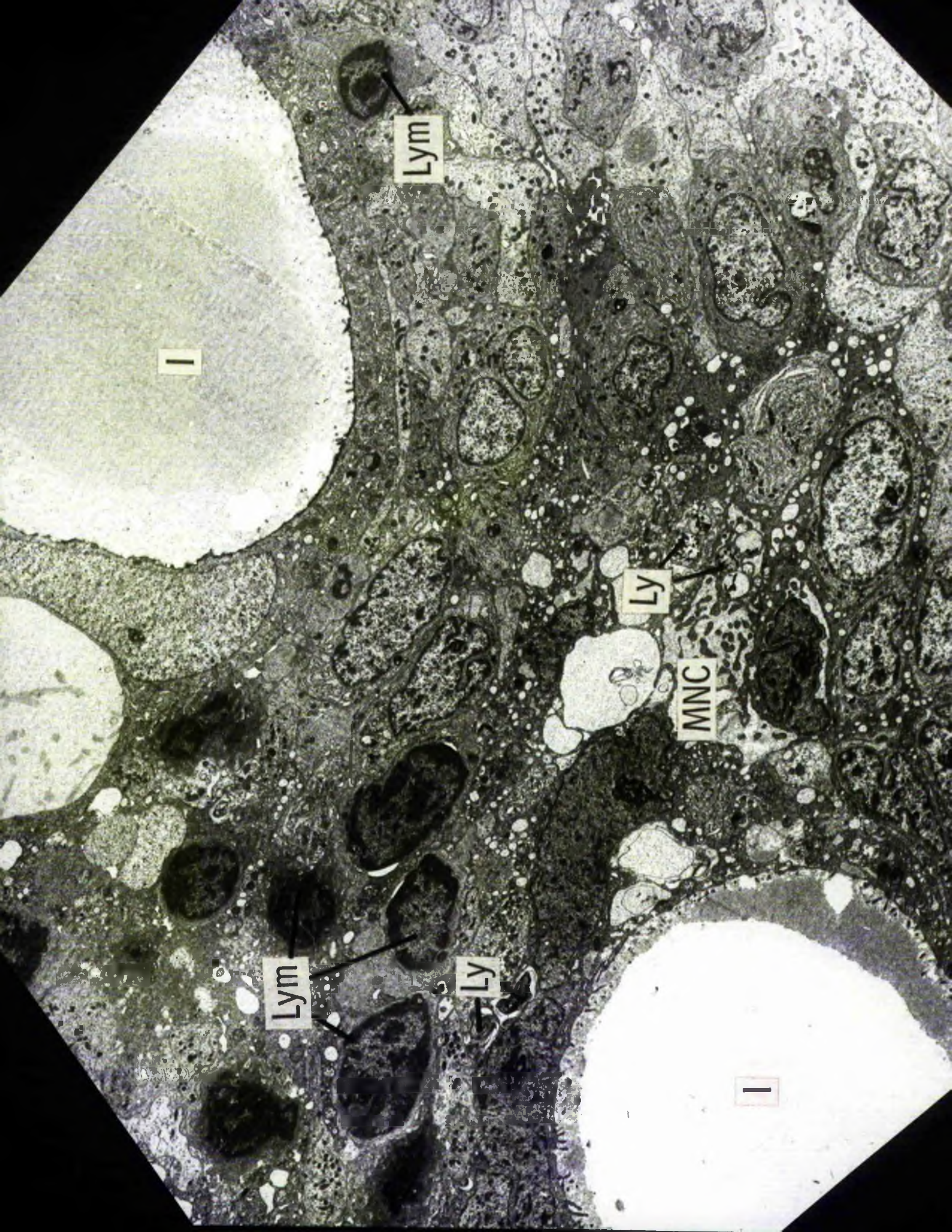
I- cytoplasmic 'inclusions'

Ly- lysosomes

Lym- lymphocytes

MNC- multinucleated cell





Lym

I

Ly

MNC

Lym

Ly

I



Figure 66(continued):

b. Rabbit oviductal epithelium showing what appears to be a degenerating  
macrophage. UA&LC. X6510

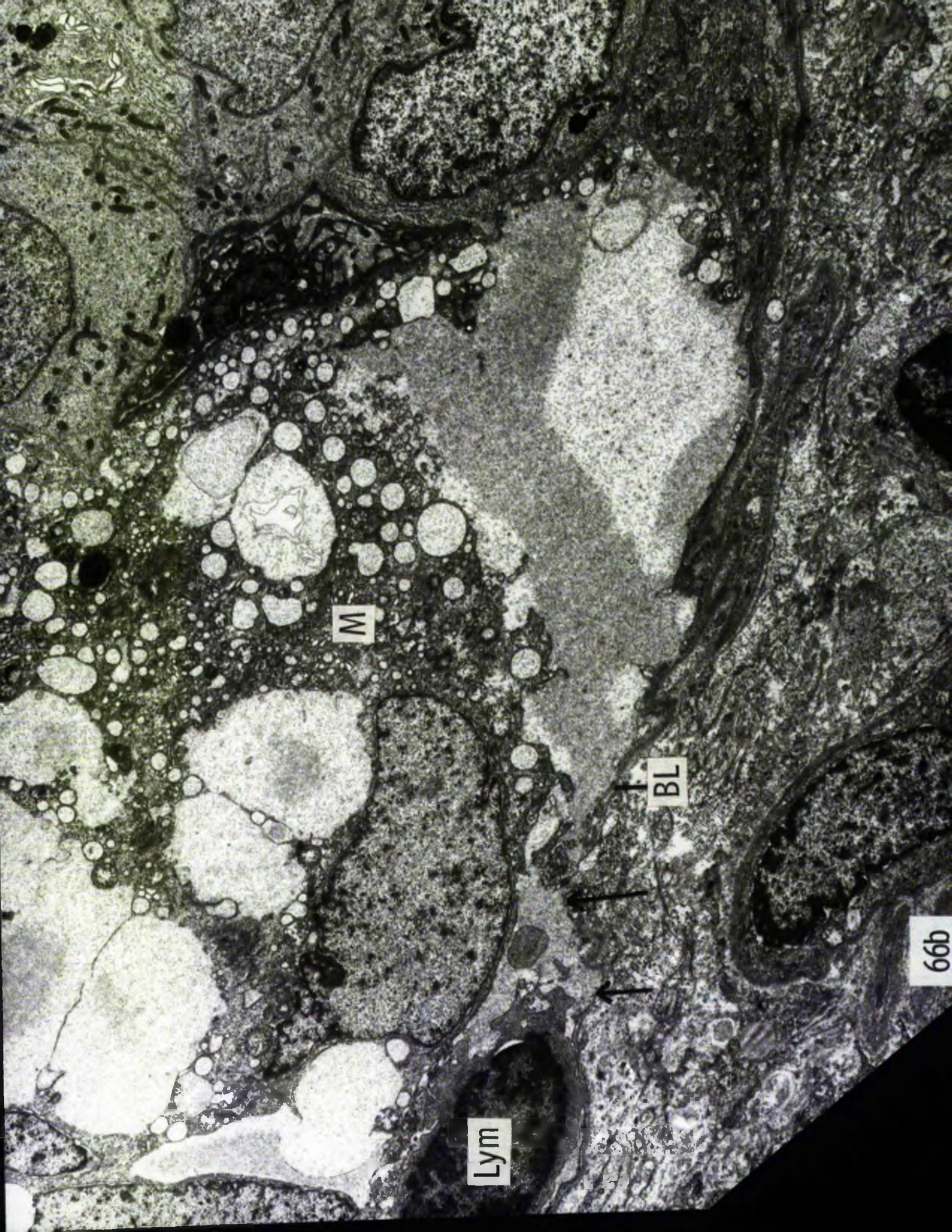
Lym-lymphocyte

M- macrophage?

BL- basal lamina

→ - gaps in basal lamina





M

BL

Lym

66b



Figure 67: Rabbit oviductal epithelium containing viral particles (at arrows)  
in the ciliated cells, some of which show degenerating cilia  
(double arrows). (Note: This is the same oviduct featured in Figs.  
65&66.) UA&LC. X3710



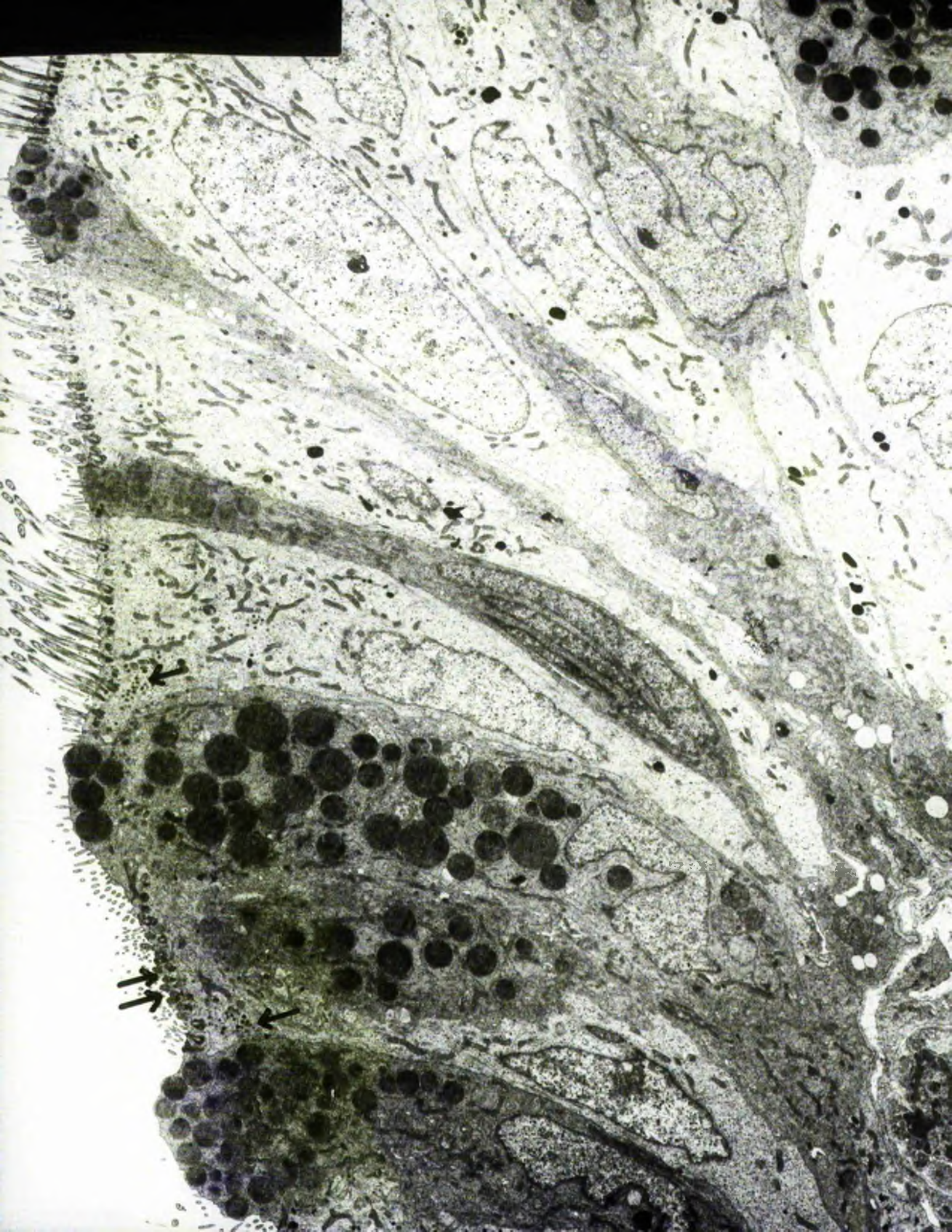




Figure 68: Viral infection of rabbit oviductal epithelium.

a. Ciliated cell containing many viral particles.

UA&LC. X23,670

b. Viral particles in a ciliated cell which also contains  
breakdown particles of basal bodies. UA&LC. X40,000

BB- broken down basal bodies

→ - what may be viral particles which have shed their  
protein capsids

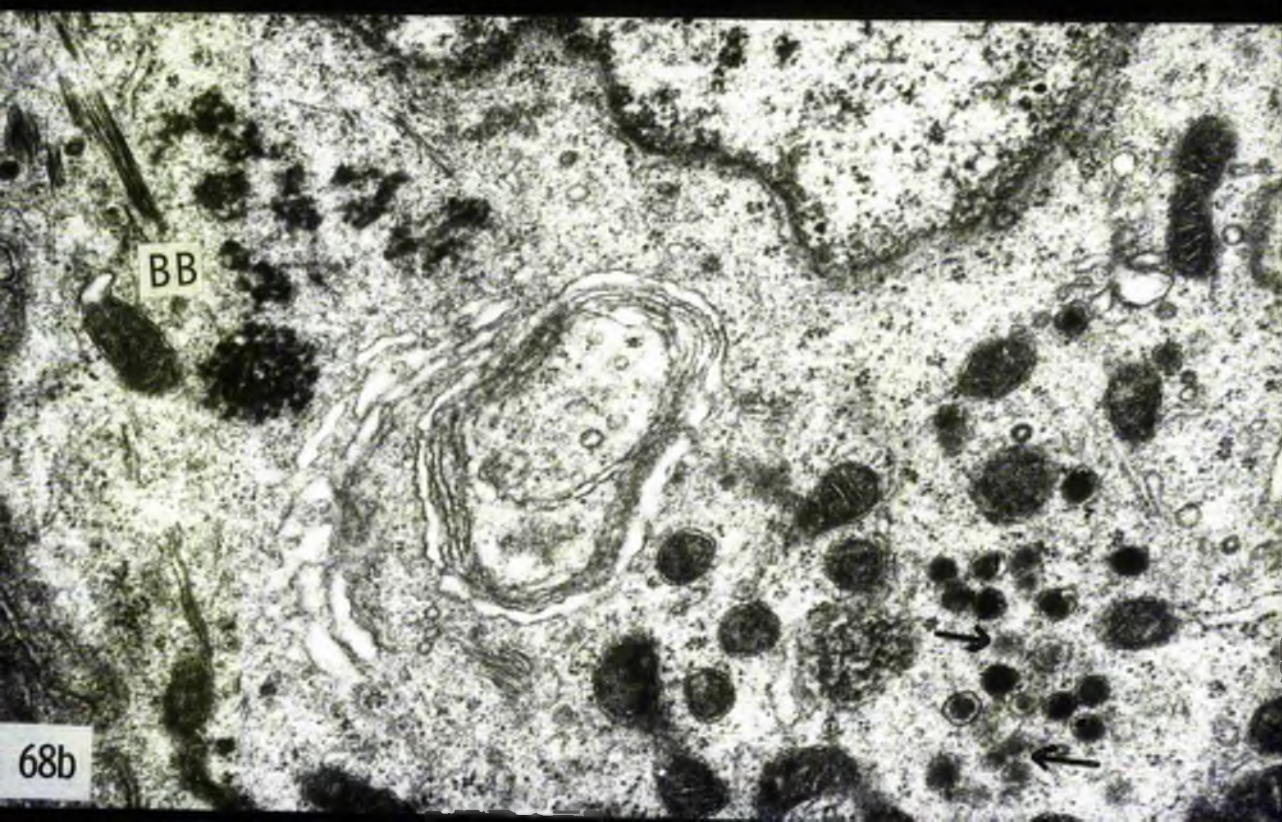
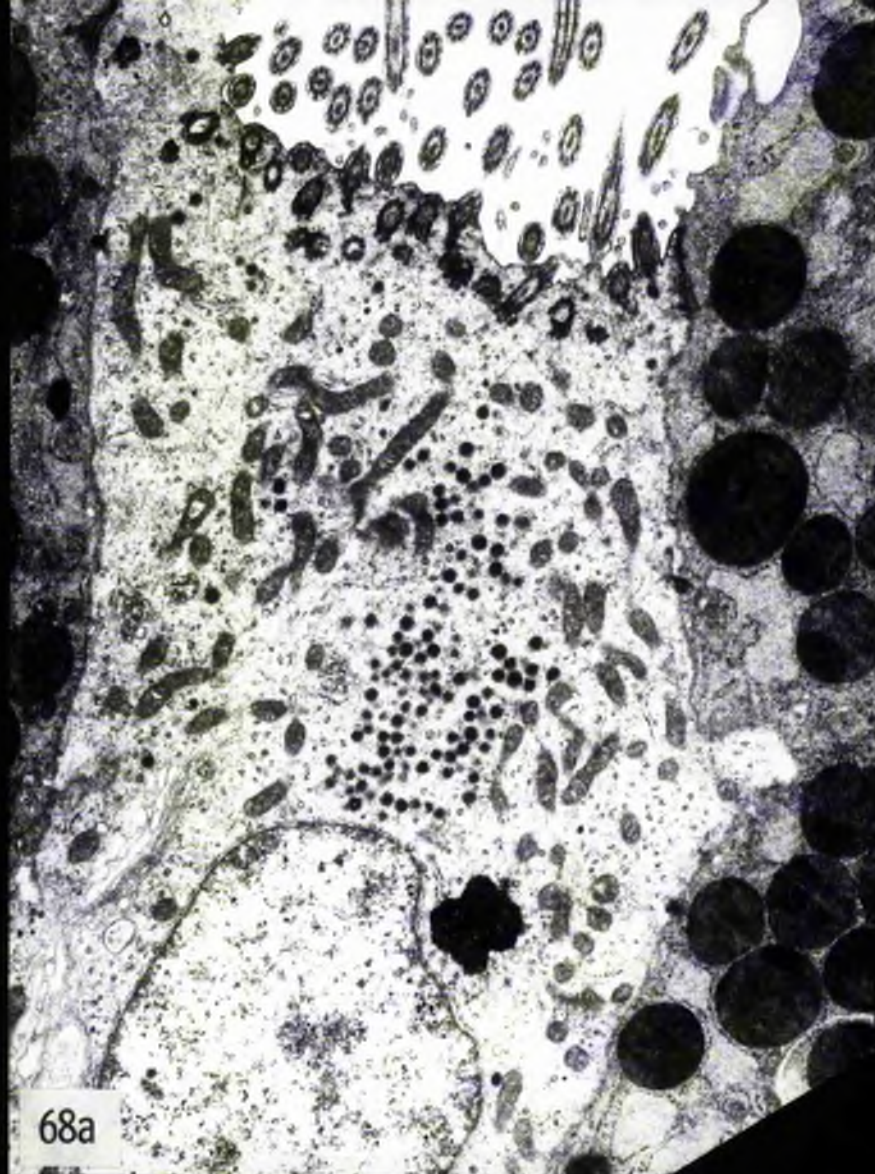




Figure 68 (continued):

c. Large collection of cytoplasmic fibrils related to a broken down basal body (at arrow) found in a ciliated cell containing viral particles. UA&LC X40,000

d. Viral particles related to the surface of a degenerating cilium (arrows). UA&LC. X40,000

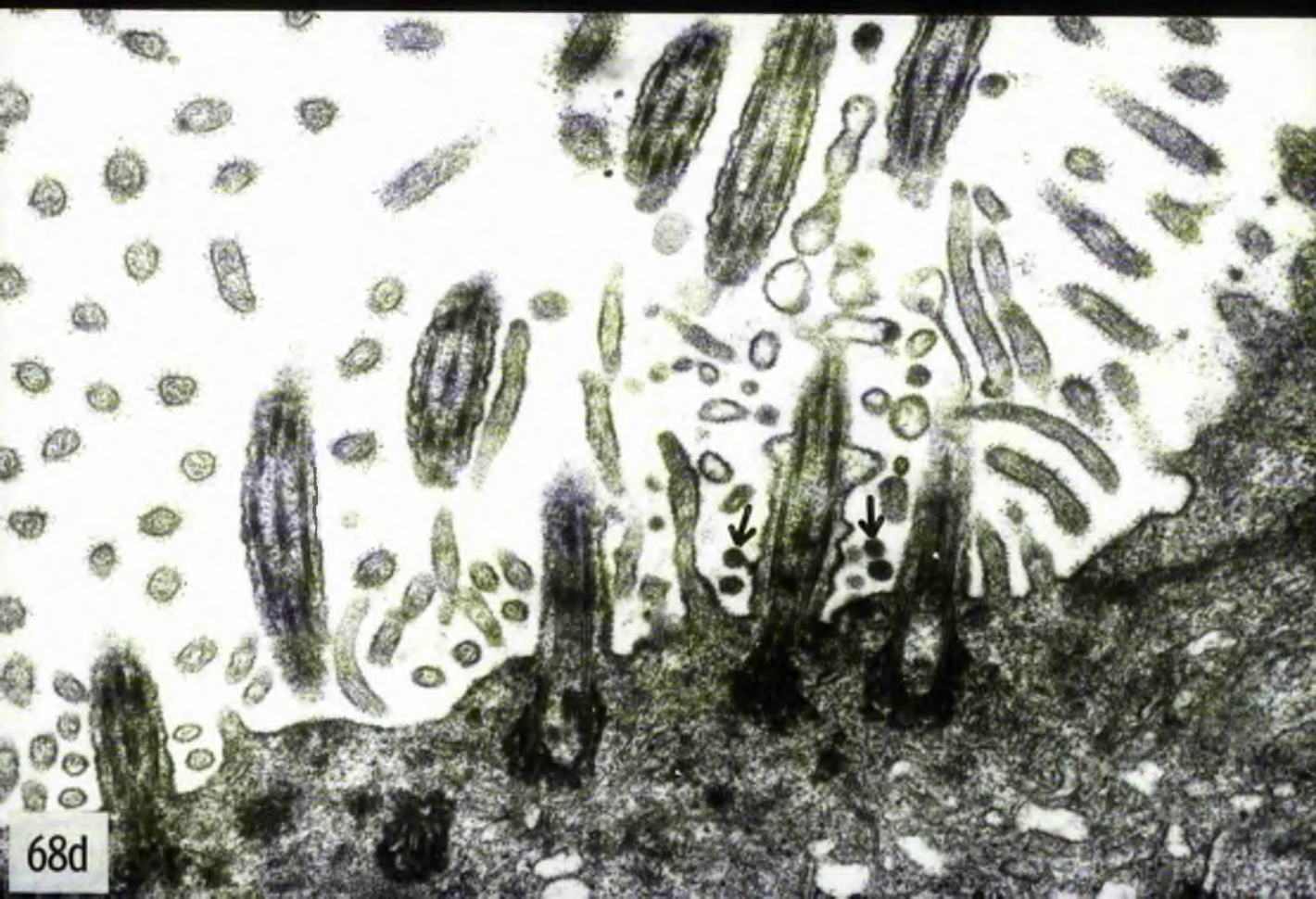
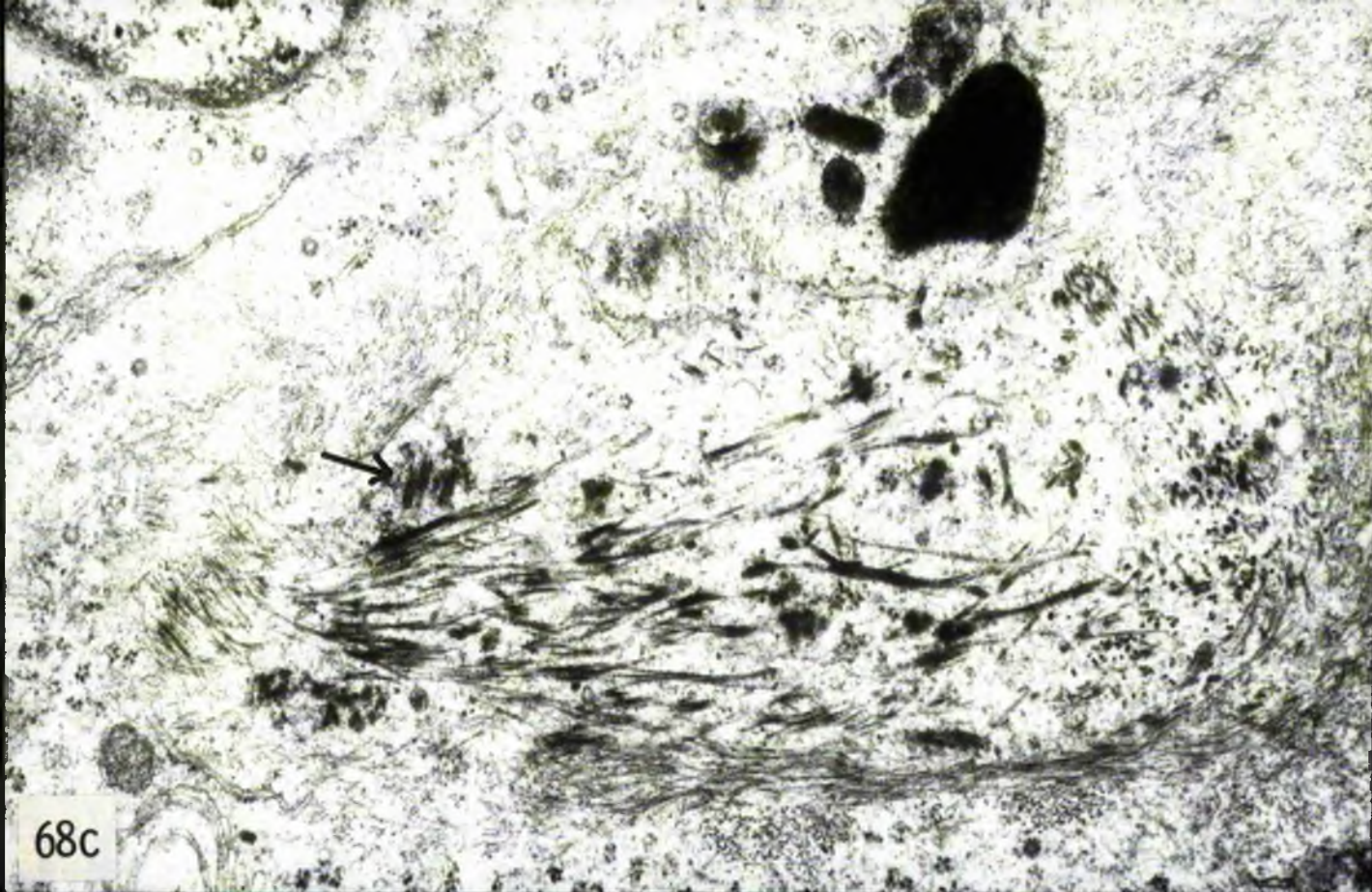


Figure 69: Rabbit oviduct showing adenomyosis. Three hundred and fifty-five days estrogen. H&E. X64

Figure 70: Neoplasm of rabbit oviduct. Two hundred and ninety-one days estrogen.

a. H&E. X64      Boxed areas indicate regions shown in detail in b, c and d.



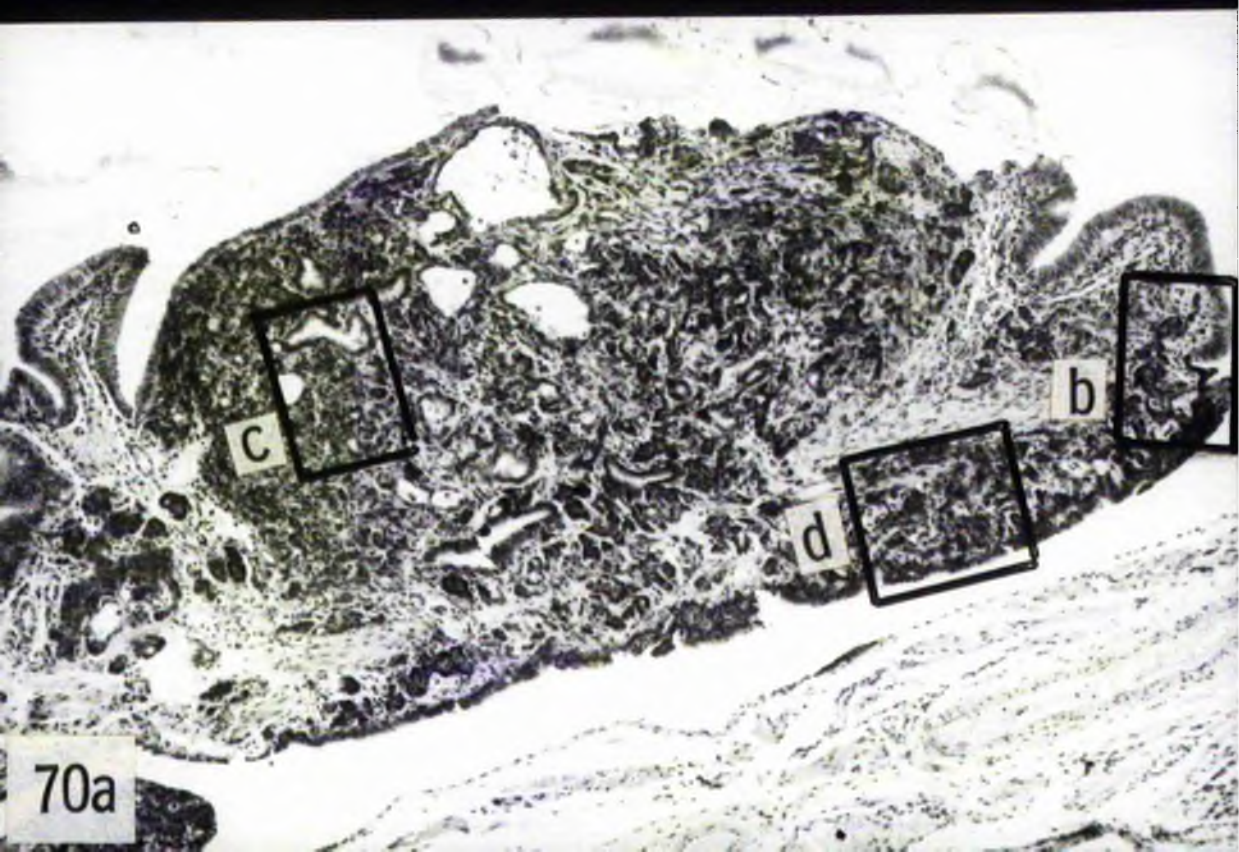
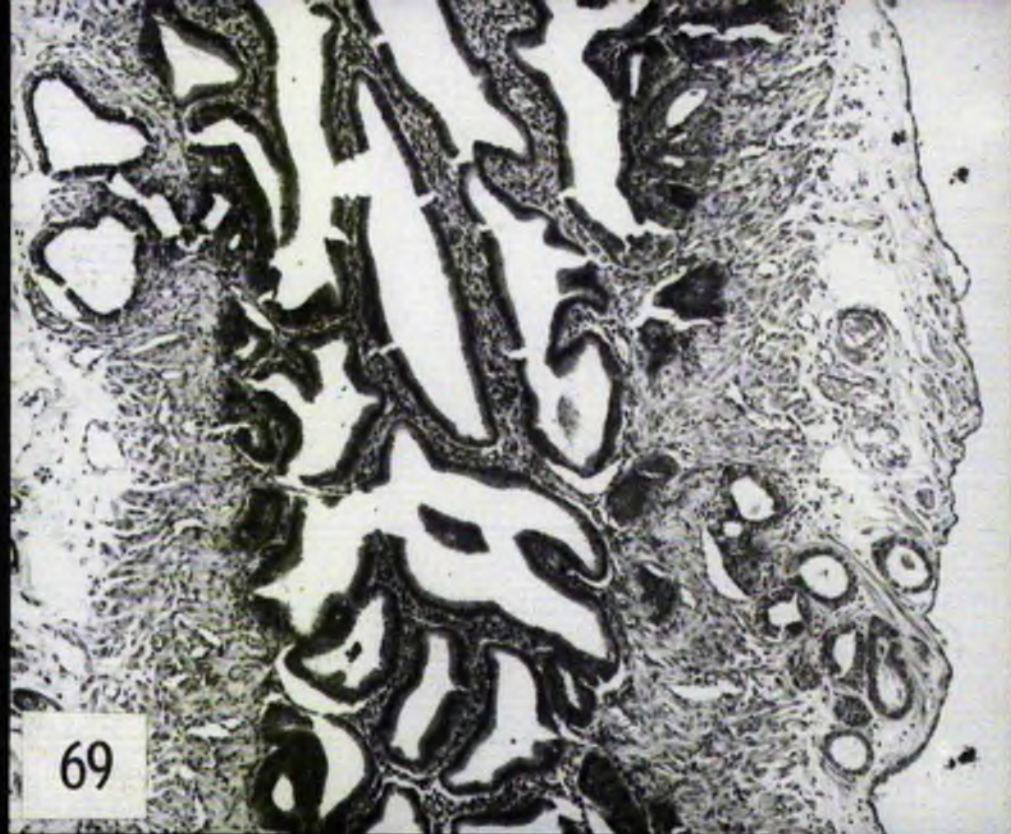


Figure 70(continued):

- b. Region showing direct transition (at arrow) between neoplastic and normal oviductal epithelium. H&E. X400
  
  
  
  
  
  
  
  
  
  
- c. Area of neoplasm showing solid clusters of anaplastic cells and also acini containing well-differentiated oviductal epithelium (arrow). H&E. X400
  
  
  
  
  
  
  
  
  
  
- d. Invasion of fimbrial oviductal epithelium by neoplastic cells. H&E. X400



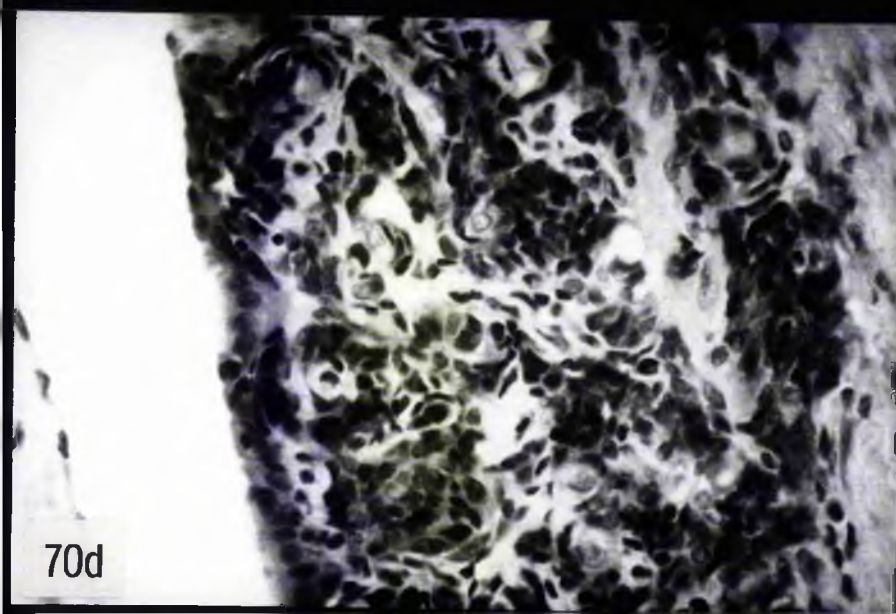
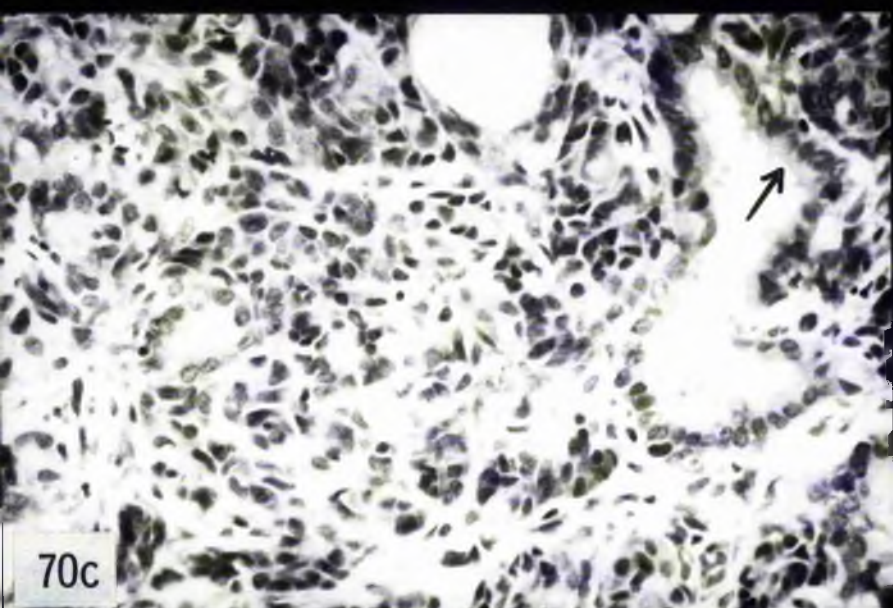
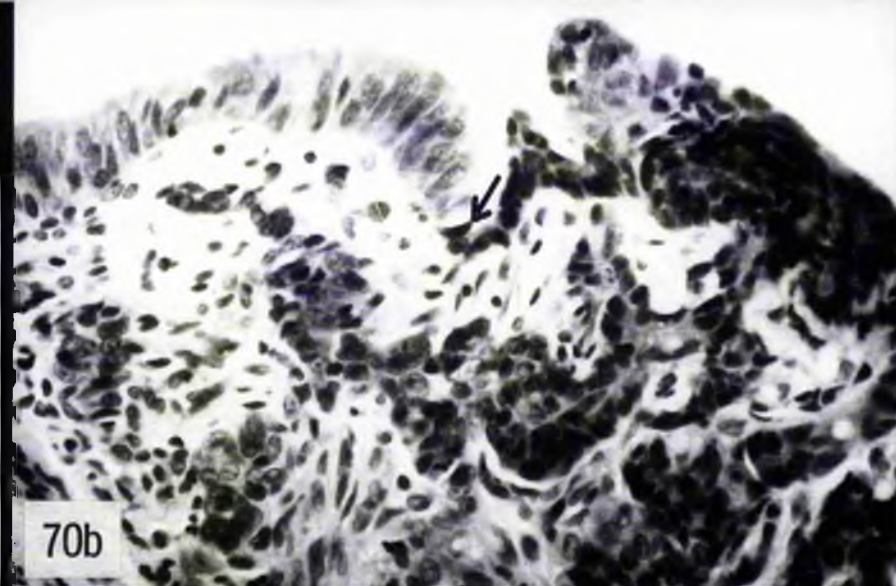


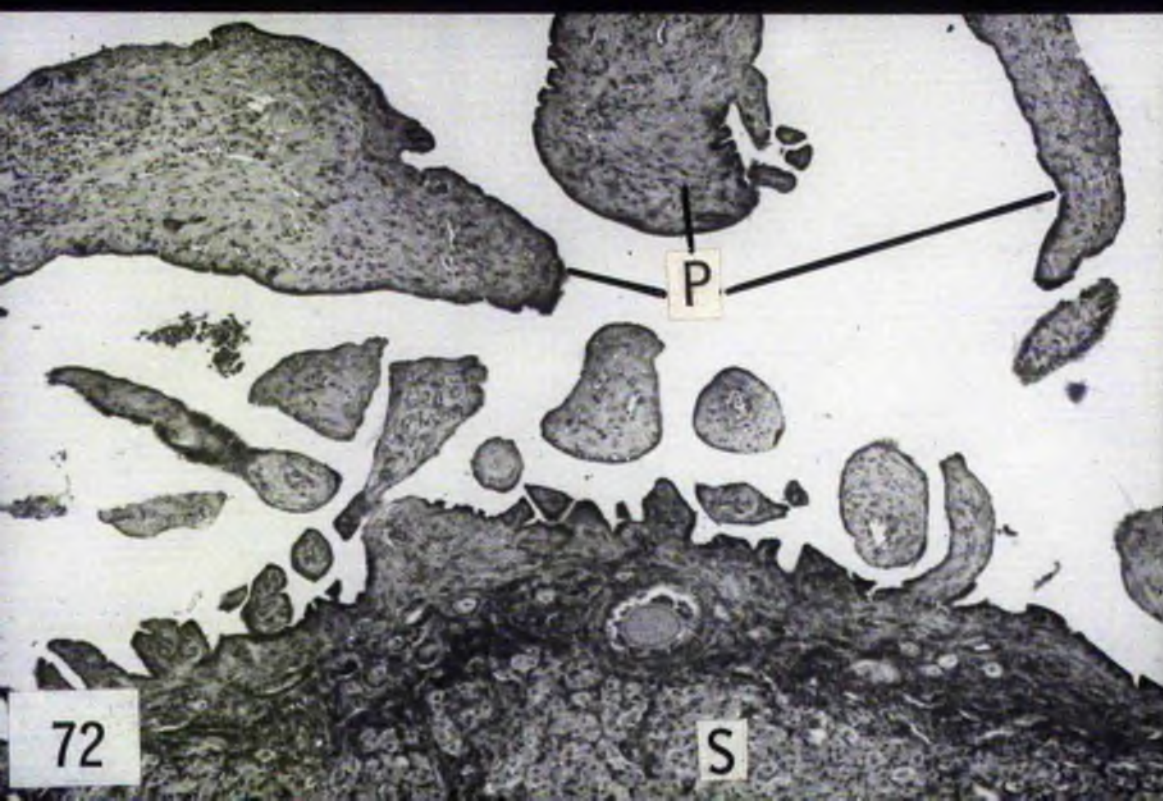
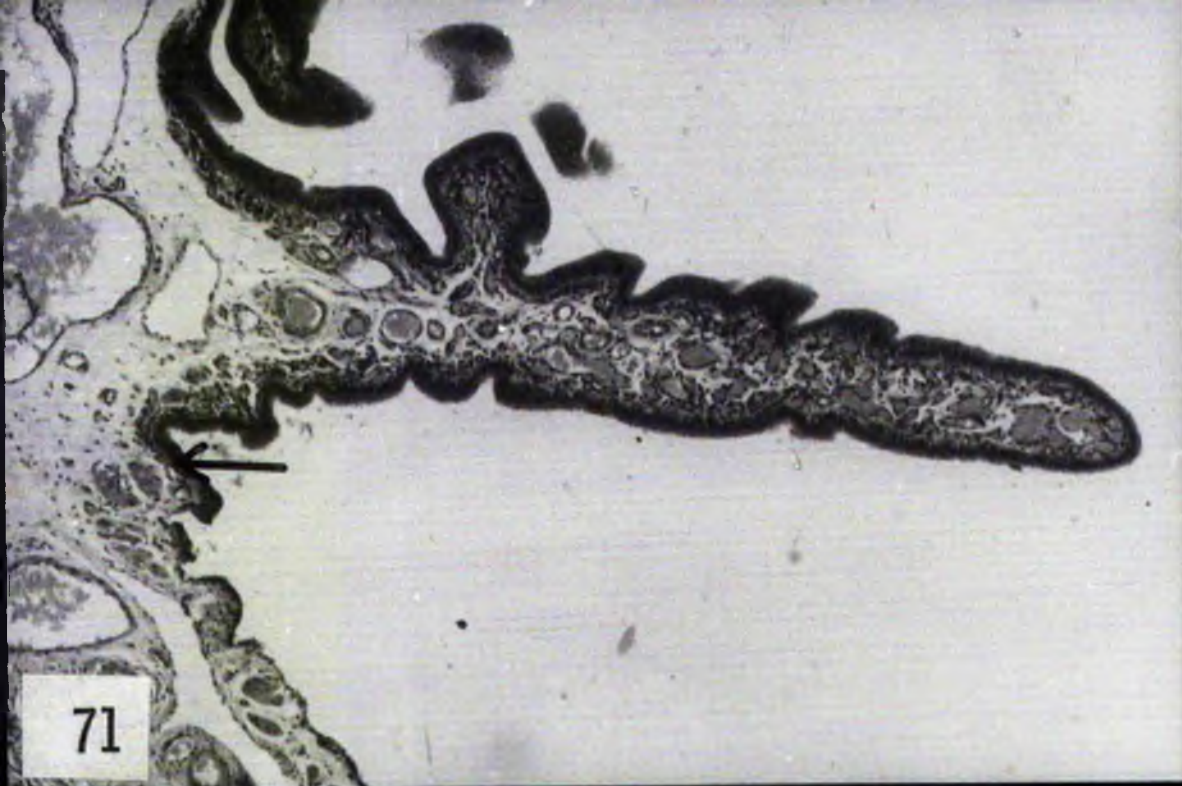
Figure 71: Fimbria of rabbit oviduct showing the transition of oviductal epithelium to serosal mesothelium (at arrow). Three hundred and fifty-five days estrogen. H&E. X64

Figure 72: Ovary of rabbit whose oviductal fimbria displayed neoplastic change. H&E. X64

P- papillary projections

S- stroma of ovary showing hypertrophy of interstitial tissue and excessive luteinization





## APPENDIX: THE RAT OVIDUCTAL EPITHELIUM IN ORGAN CULTURE

### Introduction

One of the methods available for the study of the functioning of an organ without the interference of the multitude of factors which influence it in vivo is the technique of organ culture. Isolation of a tissue in this manner allows the experimenter to exercise a great deal of control over the environment of the organ. Theoretically then, this system would be ideal in establishing the direct effects of a hormone on its target tissue, without the possible intervention and interaction of other endogenous hormones. Although ovariectomy removes the major source of estradiol, it does not eliminate all hormonal influences. To create the same conditions in vivo would mean removing the entire endocrine system of the animal. The chances for survival of such an animal are very low and the possibility of influence from extra-endocrine stores of hormones (e.g. in fatty tissue) would still remain.

Oviducts of various species have been maintained in organ culture by several researchers. Oviducts of fetal mice (Rumery, 1969a&b) and guinea-pigs (Price, Zaaijer and Ortiz, 1969) have been successfully cultured. In addition to studying the subsequent development of the fetal oviducts in culture, these authors also studied the reaction of the oviducts when explanted with the fetal gonads, and when hormones were added to the culture medium.

Fetal organs are more easily maintained in culture than those of the adult, the latter being much more dependent on an adequate supply of oxygen (Moscona, Trowell and Willmer, 1965). However,

a few investigators have used the organs of adult animals in culture to examine various aspects of oviductal physiology.

Oviducts from mature mice mated prior to explant have been maintained in organ culture on chemically defined media (Gwatkin and Biggers, 1963). In this study the development of the fertilized eggs to the blastocyst stage in the explanted oviducts was followed during the four days in culture. A later study (Whittingham, 1968a&b) examined various factors (e.g. hormonal status of the donor animal) affecting the development and survival of zygotes in cultured oviducts.

The effect that addition of hormones to the medium has on oviductal morphology and survival in culture has also been examined. Bousquet (1964) found that the epithelium of oviducts removed from both rabbits and rats in estrus were well differentiated after 6-7 days in organ culture and that both cilia and secretory granules were present. The epithelium of those cultures in which crystals of pure estrone were added to the medium seemed very dense, and the tall epithelial cells very frequently contained mucicarmine-positive granules. In contrast, the addition of crystals of pure progesterone to the cultures resulted in involution of the epithelium.

Another group of experimenters examining the mouse oviduct in organ culture found that the preservation of the epithelium was best when the oviducts were cultured with the donor animal's own ovaries (Stadnicka, Szoltys and Witkowska, 1973; Witkowska, 1973). This was true for oviducts removed from both estrus and diestrus animals. Those removed from estrus animals also fared well when 0.2mg% estradiol was added to the medium.



Organ culture of the oviduct has been found to be useful in the study of the pathology of certain diseases. Cultured human oviducts have been examined at the electron microscopic level at varying intervals following the addition of a suspension of gonococcus to the medium (Taylor-Robinson et al., 1974; Ward, Watt and Robertson, 1974). This procedure has allowed the investigators to study the interaction between the gonococci and the oviductal epithelial cells in detail, and also to elucidate several of the factors that enable the gonococci to invade the mucosal lining.

The effects of mycoplasmas (Taylor-Robinson and Carney, 1974) and various viruses (Barski, Cornefert and Wallace, 1959; Casal et al., 1970) on the oviductal epithelium have also been studied in organ culture systems. The morphology of the interaction between the epithelial cells and the pathogens was not however examined in these experiments. And in view of the finding of a viral-induced salpingitis in the rabbit oviductal epithelium (Part III), it is unfortunate that none of the studies mentioned above tested the effects of reoviruses on cultured oviducts.

#### Results of the Present Study

Explant survival was extremely variable in all of the organ culture experiments. Because of the small number of experiments performed and the erratic nature of the results it was not possible to determine whether the presence or absence of either estradiol or serum had any effect on the survival of the oviduct in culture.

All of the explants removed from animals treated in vivo with estrogen (experiments IV and V) contained inflammatory

infiltrate. In none of these explants was the epithelium preserved following culture for 2-9 days. Therefore it was not possible to determine whether the intracellular mucus inclusions and proliferative nodules that were so characteristic of the rat oviductal epithelium following lengthy estrogen treatment (see Part III) would persist following isolation from hormonal stimulation.

Explants cultured for up to nine days in medium containing estradiol did not develop either intracellular mucus inclusions or proliferative nodules. The epithelium of isthmic explants exposed to estradiol for two days showed no signs of increased levels of protein synthesis when examined with the electron microscope. In fact, the ultrastructure of these explants was indistinguishable from isthmic segments cultured for two days in medium alone. Unfortunately the epithelium of explants cultured for longer periods of time in experiment VI suffered cellular vacuolization, which was believed to be a type of fatty change (Fig. 81). Therefore it was not possible to form any conclusions concerning the subcellular effects of estradiol on the epithelium of the oviduct in organ culture.

In spite of the difficulties mentioned above, survival of several oviductal explants made it possible to examine the behaviour of the epithelium in organ culture. In many instances, the microscopic architecture of the oviduct was preserved, and it was possible to identify preampullar (Fig. 73c), ampullar (Fig. 73a), isthmic (Fig. 73b) and junctura (Fig. 76) segments of oviduct. In other explants however, the mucosal folds disappeared, and a single layer of columnar or cuboidal epithelium

lined a more or less cylindrical lumen.

In the early days of culture the epithelial cells were often observed to migrate out of the cut ends of the explant and on to the surface of the oviduct. In some cases this led to a complete layer of epithelial cells on the external surface of the explant. The majority of these epithelial cells were well differentiated ciliated and secretory cells (Fig. 73d), however undifferentiated epithelial cells were also present. These surface epithelial cells were occasionally observed to organize the underlying connective tissue into what appeared to be blunt folds.

Occasionally mitoses were observed in the luminal epithelium (Fig. 73d). One well preserved explant that had a complete external layer of epithelial cells continuous with those lining the lumen showed several epithelial mitoses per section.

The ultrastructure of the epithelial cells of the oviduct maintained in culture for 2-9 days (Figs. 74-78) did not differ markedly from that of oviducts studied immediately following removal from the animal. Both ciliated and granulated nonciliated cells were seen throughout the nine day culture period.

There was still evidence of protein synthesis following nine days in culture (Fig. 77). However in the later days of culture the morphology of the secretory granules seemed to change from that seen in the normal rat (Fig. 5b) and also seen in the earlier stages of culture (Figs. 75 & 83a). In some instances the granules looked like vacuoles containing a dark core (Fig. 76), whereas in others the size and density of the granules were reminiscent of the small mucus granules found in the undifferentiated cells of the immature rat's oviduct (Fig. 77).

Cells bearing well formed cilia were observed throughout the nine day culture period (Figs. 77 & 78). Although no cells were observed in the actual process of cilium formation, one cell contained what appeared to be a collection of proliferative elements (Fig. 78-PE). Segments of isthmus seemed to last longer in culture than explants taken from other regions, and therefore the majority of observations were made on this part of the oviduct. As ciliogenesis occurs mainly in the preampullar segment of the oviduct where ciliated cells predominate, it was not surprising that more examples of this process were not found.

A large collection of secondary lysosomes and residual bodies suggested that the epithelial cells of the oviduct in culture had a higher than normal rate of autophagic breakdown of cytoplasmic organelles (Figs. 74 & 77-Ly). In some areas containing several dying cells, healthy neighbouring cells were observed to phagocytize bits of the necrotic cells (Fig. 79). The dead material was sequestered in giant isocytophagic lysosomes (Fig. 80-GIL). These observations confirmed the speculation that the epithelial cells of the oviduct were capable of digesting large amounts of material in a variety of types of giant lysosomes (see Fig. 7-GAL and Fig. 54-GLL).

Although morphologically 'normal' epithelium could be found in oviductal explants throughout the nine day culture period, it was also observed to undergo various alterations. In some instances the epithelial cells appeared to lose their polarity, and occasionally assumed a horizontal, stratified position (Fig. 77). In most cases typical intercellular junctions were preserved (Fig. 74-JC). However, in other situations the adjacent lateral cell membranes

were separated by a dense substance within the intercellular space, and in some places direct apposition of the two membranes appeared to obliterate the space (Fig. 81).

One of the most frequently encountered changes was a vacuolization of the epithelial cells that on electron microscopic examination appeared to be a type of fatty change. Lipid droplets are not uncommonly found in the epithelium of the normal rat oviduct (Fig. 7-L). However the number of lipid deposits observed in the epithelium of cultured oviducts was greatly increased (Figs. 76 & 77-L) and eventually reached the point where they seemed to interfere with the normal functioning of the epithelial cells (Figs. 82). The epithelium in these instances was flattened, possessed rudimentary microvilli and did not exhibit any signs of synthesis of protein for export. Although fat droplets occupied a large proportion of the cells' cytoplasm, the cells themselves did not appear to be undergoing necrosis.

One of the main objectives of the technique of organ culture when used to study the physiology of a fully differentiated tissue is to preserve the status quo as regards the immediate environment of that tissue in a given organ. Dissociation of the tissue from its neighbours (e.g. epithelial cells isolated in cell culture) usually leads to dedifferentiation of the tissue (see Grobstein, 1959). Electron microscopic examination of the oviductal explants in the present experiment revealed that in many instances the normal relationship between the epithelial cells and the underlying lamina propria had been disturbed.

One of the first signs of this was an apparent degeneration of the lamina propria, which was visible at the light microscope



level (Fig. 73b). Connective tissue cores of epithelial folds appeared to be 'collapsed' (Fig. 78-IP). The epithelial basal lamina was often either missing (Figs. 83 & 84), or greatly thickened (Fig. 84). In many cases pseudopodia from the basal aspect of the epithelial cells could be seen passing into the subjacent lamina propria (Figs. 83b & 85). In other instances where the basal lamina was missing direct contacts were established between epithelial and connective tissue cells (Figs. 83 & 84).

De-differentiation of the epithelium in the oviductal explants occurred with greater frequency as the time in culture increased. This was especially obvious in explants where the connective tissue element had undergone extensive degeneration. The epithelial cells could not be distinguished as either secretory or ciliated elements (Fig. 86). They were usually considerably flattened and the nuclei were very pleomorphic. The cell surfaces possessed very short, regular microvilli which often appeared to be 'beaded' due to the budding of minute cytoplasmic processes (Fig. 86-arrows).

#### Comment

Cavazos and Lucas (1970,1973) reported the appearance of large vacuoles of a lysosomal nature in the epithelial cells of the human endometrium. They described three different types of 'giant' lysosomes: autophagic, leukophagic and isocytophagic. 'Giant' lysosomes have also been observed in the human oviductal epithelium (Horbelt, 1970).

Large lysosomes of an apparently autophagic nature were observed in the present study in the oviductal epithelium of

the normal rat (Part I, p.51). Cavazos and Lucas (1970) believe that this type of giant lysosome represents an area of focal cellular degeneration, and that following degradation the contents are eventually re-incorporated into the cytoplasm. In Part III of this study, giant lysosomes observed in the epithelial cells of the rat oviduct were believed to contain the remains of degenerating leukocytes, which were present in the epithelium in large numbers as a result of inflammation.

According to Cavazos and Lucas (1970), 'isocytophagosomes' are formed when the remains of a cell which has died in situ are taken up by the neighbouring cells and sequestered in giant lysosomes. The rate of cell turnover in the normal oviductal epithelium is very low, and effete, dying cells are seen only rarely. However in some of the organ cultures which were examined with the electron microscope cell necrosis was fairly common. In these regions of the explant, several 'healthy' cells could be observed in the process of surrounding, and eventually engulfing, bits of dead tissue in large vacuoles, thus demonstrating quite convincingly that the oviductal epithelial cells of the rat are also capable of 'giant' lysosome formation.

In many of the oviductal explants the epithelial cells retained their functional specificity for several days. However cells with small mucus granules resembling the 'undifferentiated' cells of the immature rat oviductal epithelium began to appear among the ciliated and secretory cells during the later stages of culture. Following nine days in culture the epithelial cells of most of the explants had lost their differentiated characteristics.

Prior to the actual 'de-differentiation' of the epithelial cells however, several observations revealed that the essential homeostatic mechanisms in the oviduct had been disturbed. An 'abnormal' proliferation of the epithelium, indicated by the presence of several mitotic figures (which as mentioned previously are virtually absent in the 'normal' oviductal epithelium), occurred in some explants. This was originally thought to be due to the outward migration of epithelial cells on to the surface of the explant. However Trowell (1959) observed epithelial migration from the cut ends of all of his tubular organ (i.e. ureter, ductus deferens, uterus, trachea) explants, but rarely saw mitoses. And in the present study mitoses were not detected in all of the explants showing epithelial migration.

It has been demonstrated that it is the epithelial, rather than the connective tissue elements which synthesize the basal lamina. Pierce and Nakane (1969) have shown that pathological stimulation (e.g. x-rays and bacteria) will cause epithelial cells to synthesize increased amounts of the material seen in the electron microscope as the 'basal lamina'. In Part III of the present study, duplication of the basal lamina was observed in cases of acute inflammation of the mucosa. The epithelial cells of the oviduct in organ culture, however appeared to lose the ability to synthesize this material. In several regions there was no basal lamina separating the epithelium and the connective tissue and epithelial cells were observed in direct contact with the cells of the lamina propria. In some areas pseudopodia from the epithelial cells extended through gaps in the basal lamina into the subjacent tissues.

These observations are particularly interesting in view of the disturbances in tissue interaction that occur during the development of malignant tumours. The breakdown in basal lamina synthesis with pseudopodia formation and eventual cell migration into the lamina propria seen in the present study is very similar to observations made during the experimental induction of cancer in animals (Smith, 1972) and during the examination of various human premalignant and malignant lesions (Frithiof, 1972; Sugar, 1972).

The organ culture experiments described in this Appendix are really only pilot studies. The fact that differentiated epithelial cells persisted in culture for several days confirmed that this could be a useful technique for the study of the hormonal regulation of the functioning of the 'adult' (i.e. differentiated) cells.

It also would be interesting to inoculate organ cultures of rat and rabbit oviduct with reoviruses to compare the reaction of the epithelium in vitro to that of the epithelium in vivo to this pathogen (Part III). Taylor-Robinson et al. (1974) found that although Neisseria gonorrhoeae multiplied in organ cultures of rabbit oviduct, they did not attach to the epithelium or penetrate into the cells (c.f. the behaviour of these microbes in organ cultures of human oviduct). Gonococci injected into the oviducts of living rabbits (which were ligated at both ends) however disappeared rapidly and did not multiply. This indicated that immunological defense mechanisms were preventing the multiplication in vivo, and in view of the observations made in the present study (i.e. influx of lymphocytes and macrophages into areas of viral-infected epithelium) it would be interesting to observe how the rabbit oviductal epithelium coped with reovirus

infection in vitro.

The importance of the interplay between the local tissue factors and the hormonal stimuli reaching the epithelial cells of the reproductive tract in regulating the behaviour of the cells and the determination of their functional differentiation was discussed in some depth in Part III of this report. The method of organ culture is valuable in that it allows the experimenter to control and quantify the hormonal milieu. However the observations recorded in this Appendix indicate the difficulty in preserving the status quo as regards the homeostatic interaction between the epithelial cells and the neighbouring connective tissue. Until methods are developed to substantially improve the viability and functional integrity of the connective tissue elements in organ culture systems, extreme caution must be used in the interpretation of results from in vitro experiments.



FIGURES 73--86

Figure 73: Rat oviduct in organ culture. (Note: Figures in parentheses indicate experiment no.-see Materials and Methods, pp. 45-47.)

a. Ampulla. Six days in culture (V-medium only).

H&E. X64

b. Isthmus. Two days in culture. (VI-medium+serum+estradiol). Toluidine Blue. X160

Note: Collapse of the lamina propria.

c. Preampulla. Two days in culture (V-medium +estradiol).

H&E. X64

D. Isthmus. Six days in culture (II-medium+serum).

H&E. X400

L- lumen

S- secretory cells on surface of explant

→ - mitosis

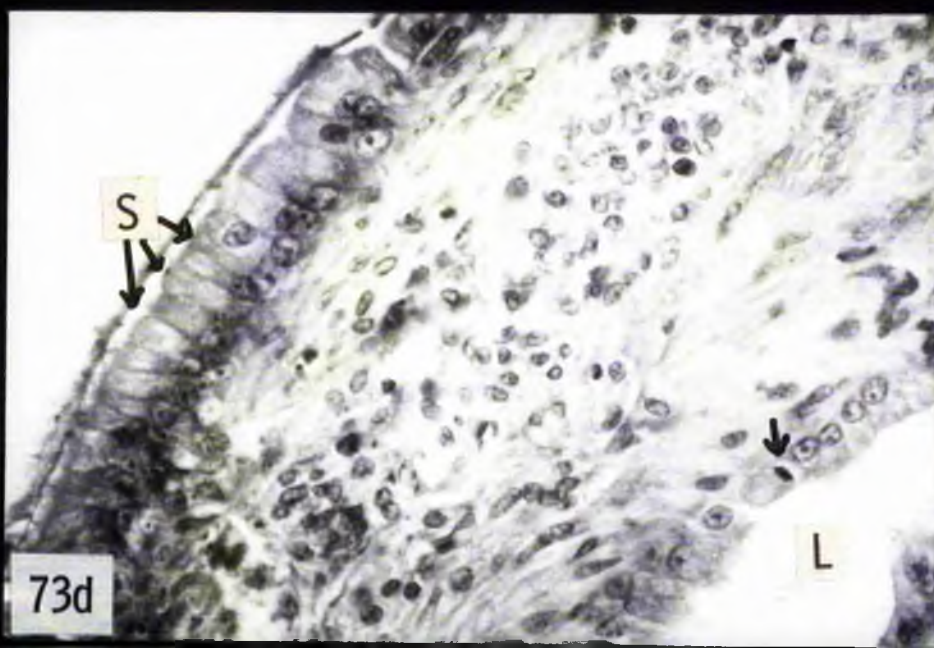


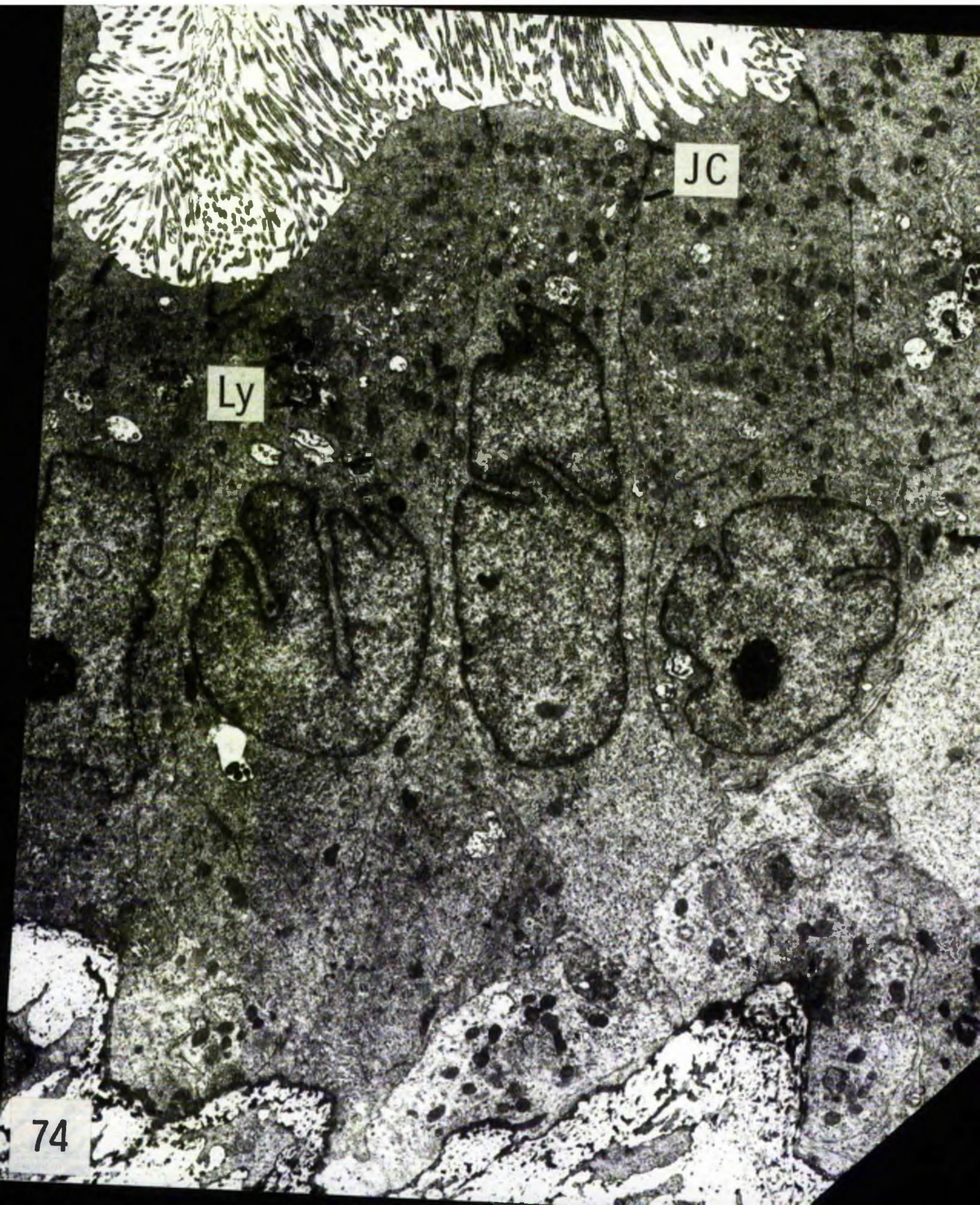
Figure 74: Rat oviductal epithelium (isthmus) in organ culture.

Two days in culture (III-medium+serum). UA&LC. X6510

JC- junctional complex

Ly- lysosomes





JC

Ly

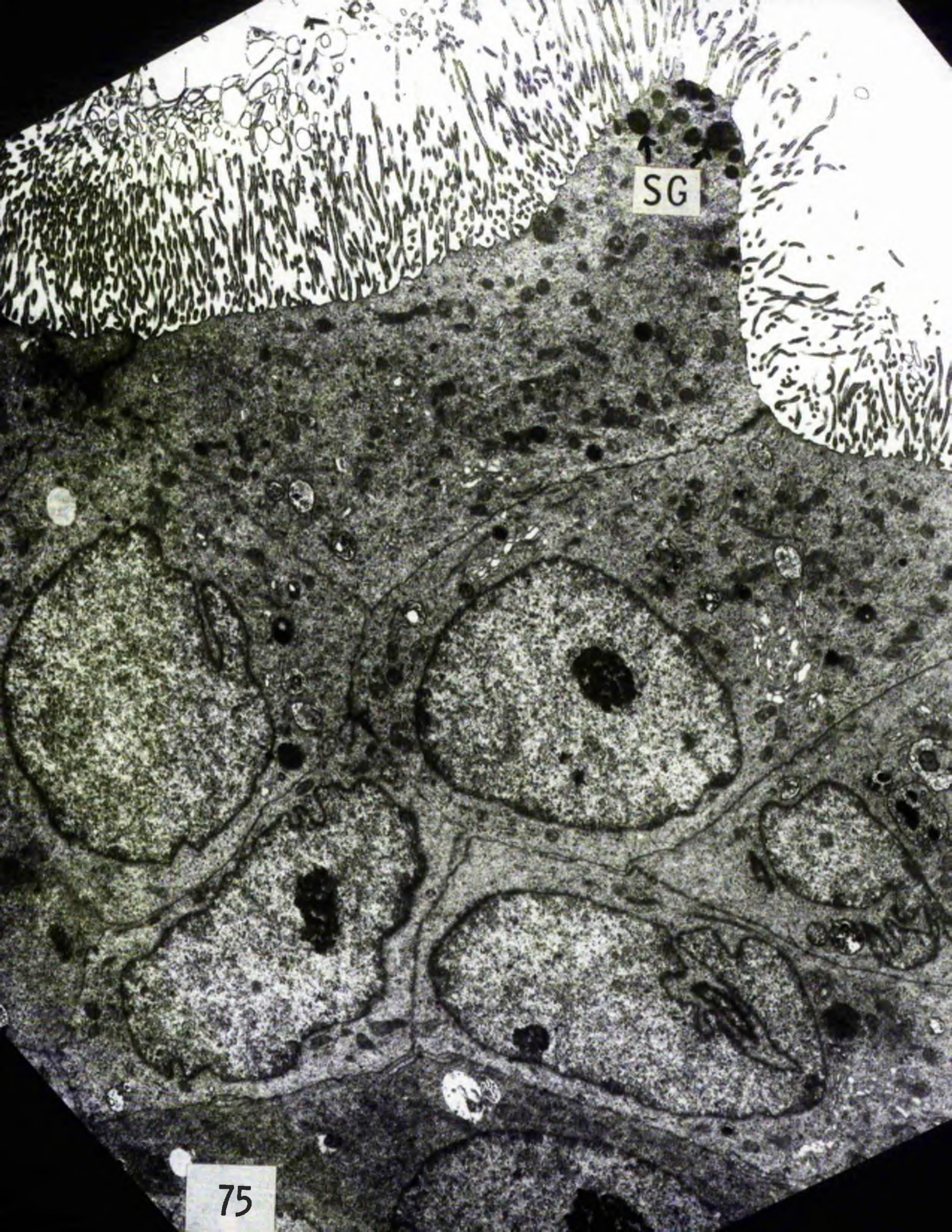


Figure 75: Rat oviductal epithelium (isthmus) in organ culture.

Two days culture (III- medium+serum). UA&LC. X6510

SG- secretory granules





SG



Figure 76: Rat oviductal epithelium (juntura) in organ culture. Six days

in culture (III-medium+serum). UA&LC. X3710

L- lipid droplets

SG- secretory granules

→ - granules with dense core



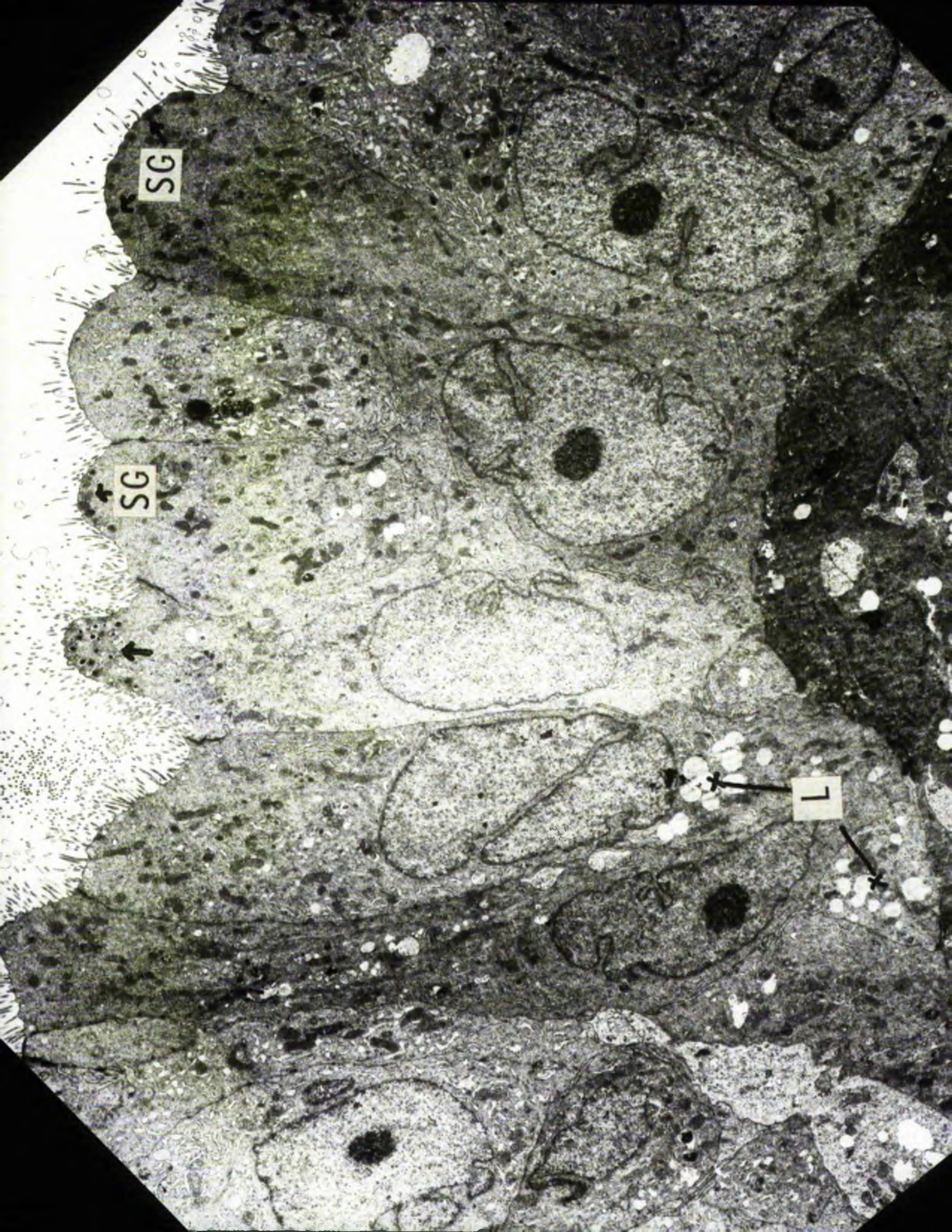




Figure 77: Rat oviductal epithelium (ampulla) in organ culture.

Nine days in culture (III-medium only). UA&LC. X6510

L- lipid droplets

Ly- lysosomes

→ - small mucus granules similar to those found in the  
undifferentiated cells of the oviduct of the immature  
rat



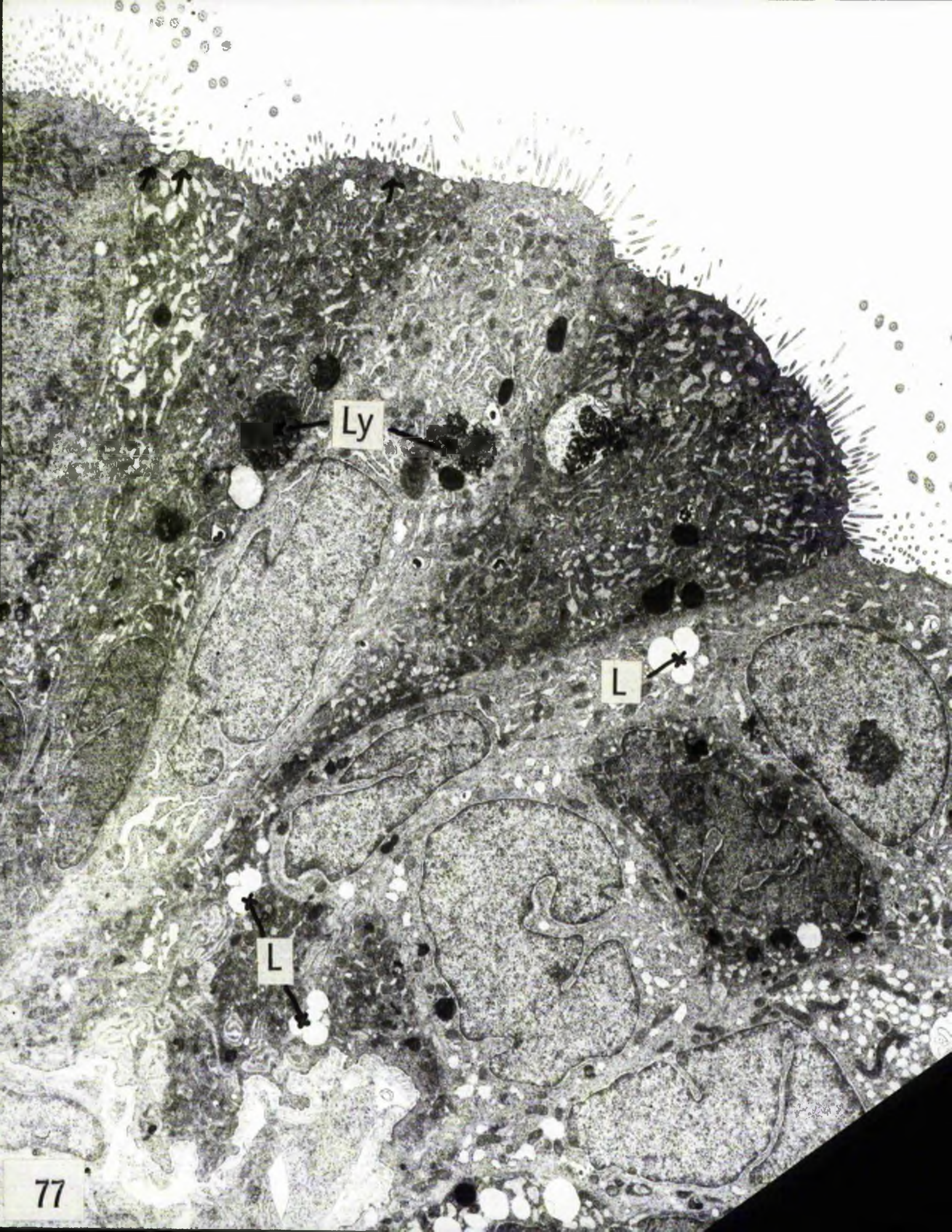




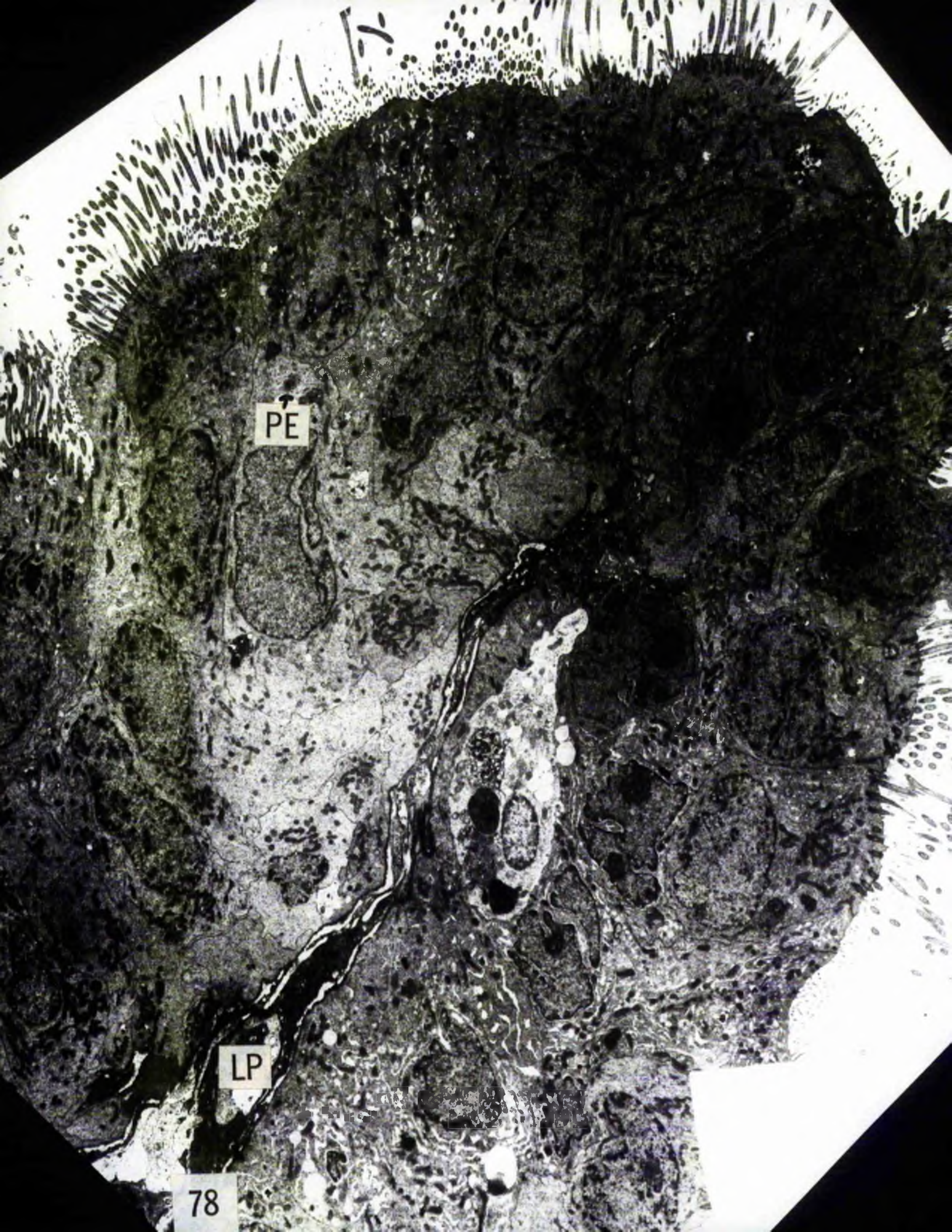
Figure 78: Rat oviductal epithelium (ampulla) in organ culture.

Four days in culture (III-medium only). UA&LC. X3710

LP- lamina propria

PE- proliferative elements





PE

LP



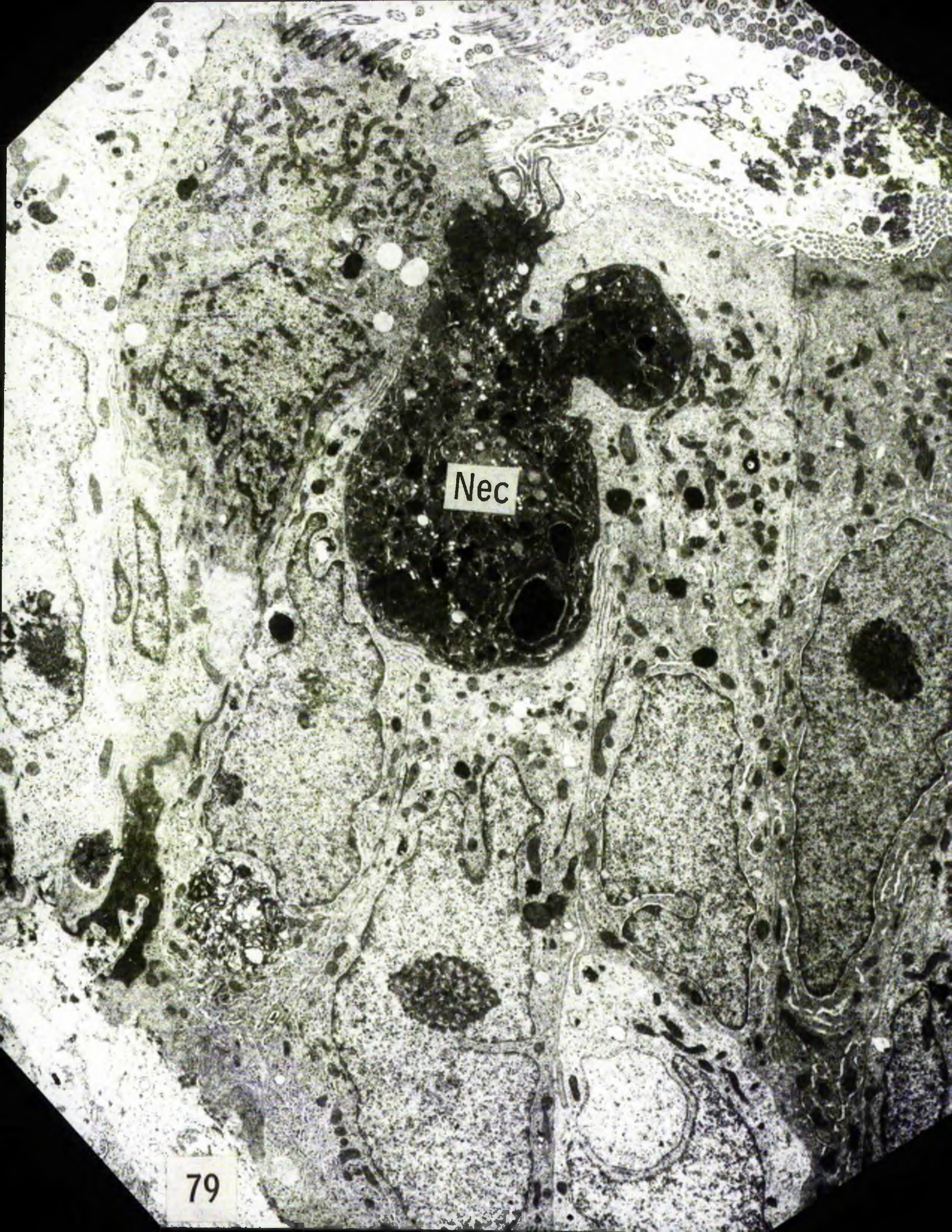
Figure 79 : Rat oviductal epithelium (ampulla) in organ culture.

Two days in culture (VI- medium+serum+estradiol).

UA&LC. X6510

Nec- necrotic cell





Nec



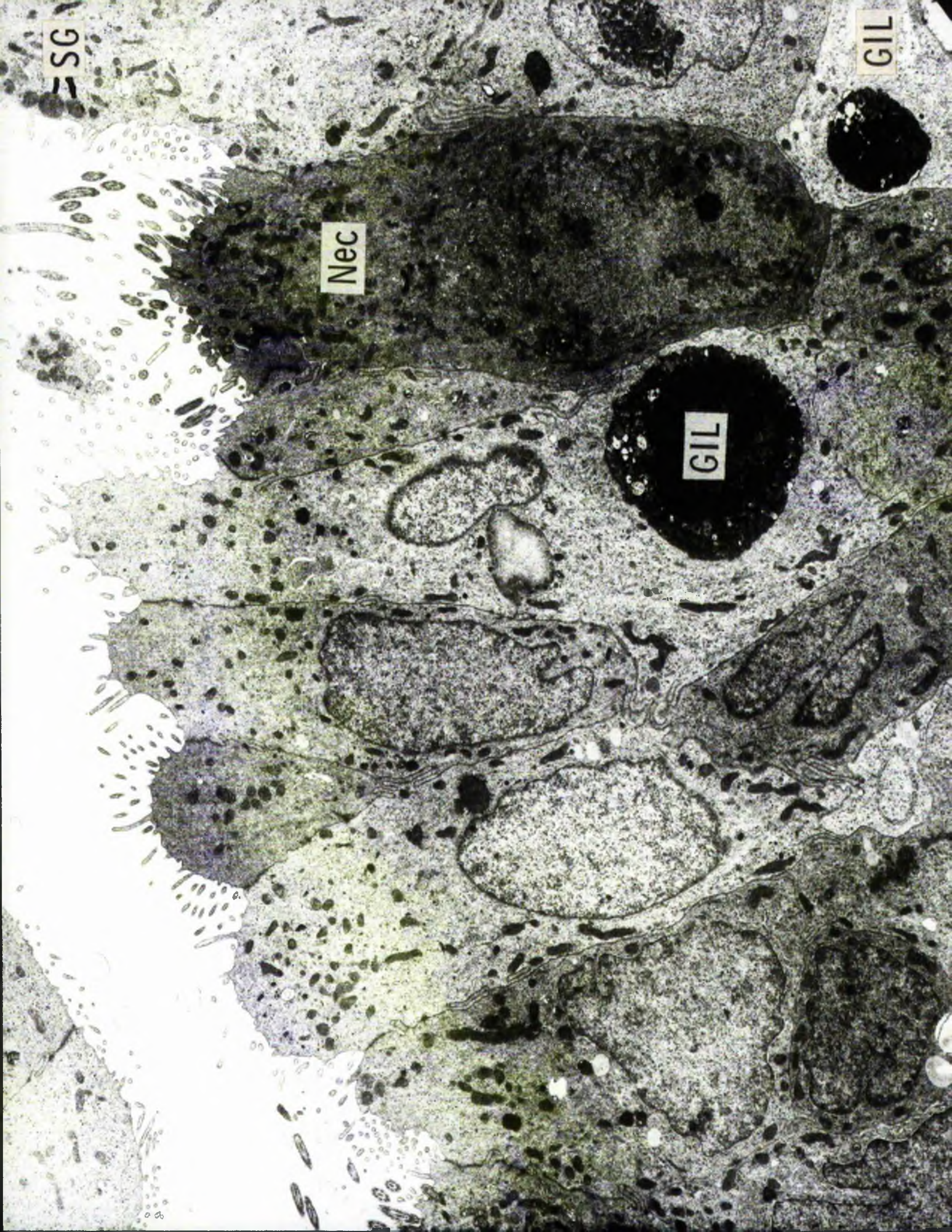
Figure 80: Rat oviductal epithelium (ampulla) in organ culture. Two days  
culture (VI-medium+serum+estradiol). UA&LC. X6510

GIL- giant isocytophagic lysosome

Nec- necrotic cell

SG- secretory granules





SG

Nec

GIL

GIL



Figure 81: Rat oviductal epithelium in organ culture. Two days  
in culture (VI-medium+serum+estradiol). UA&LC. X40,000  
ics- intercellular space

Figure 82: Rat oviductal epithelium in organ culture showing  
fatty change in the epithelial cells. Two days in  
culture (VI-medium+serum). UA&LC. X5580

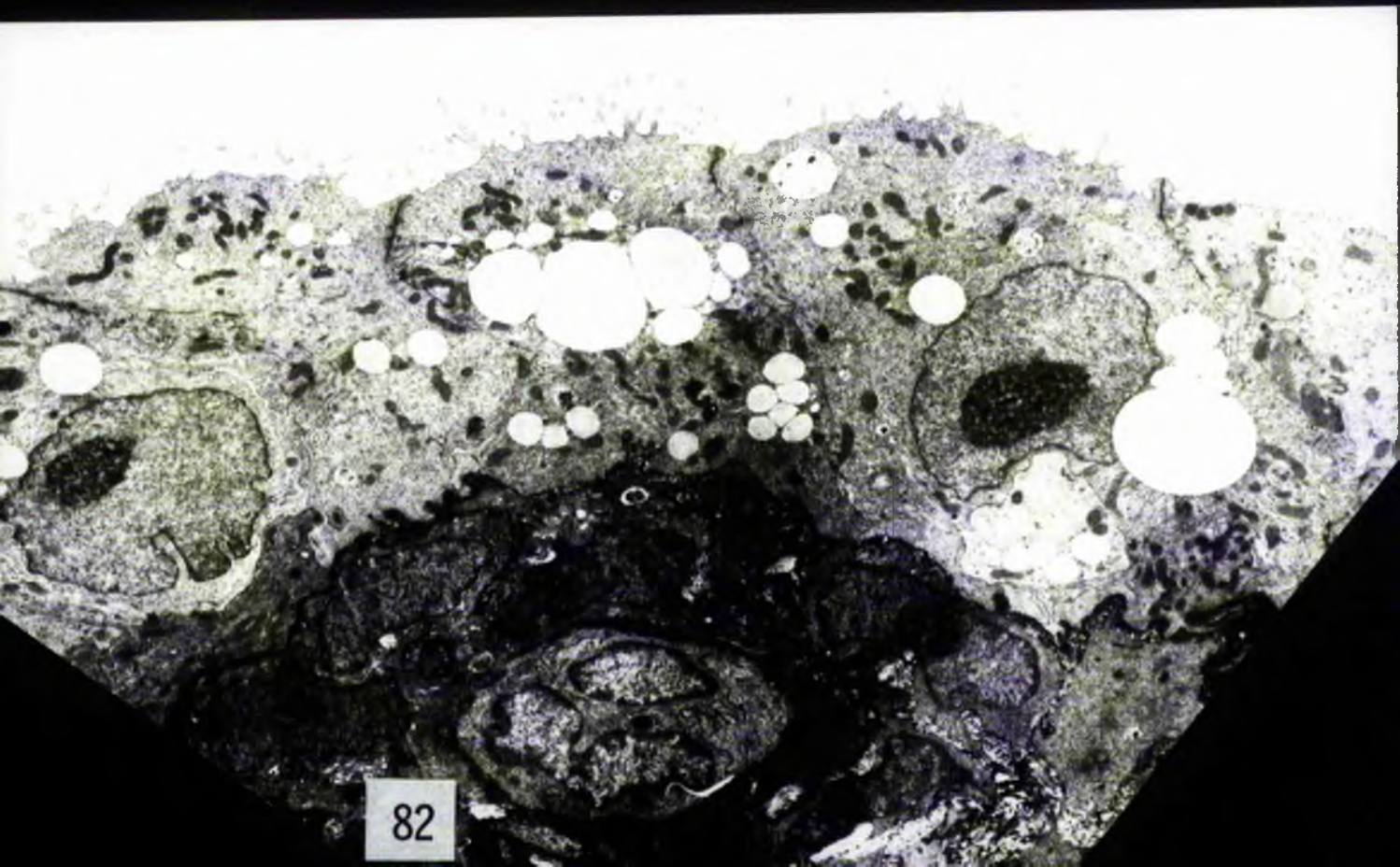
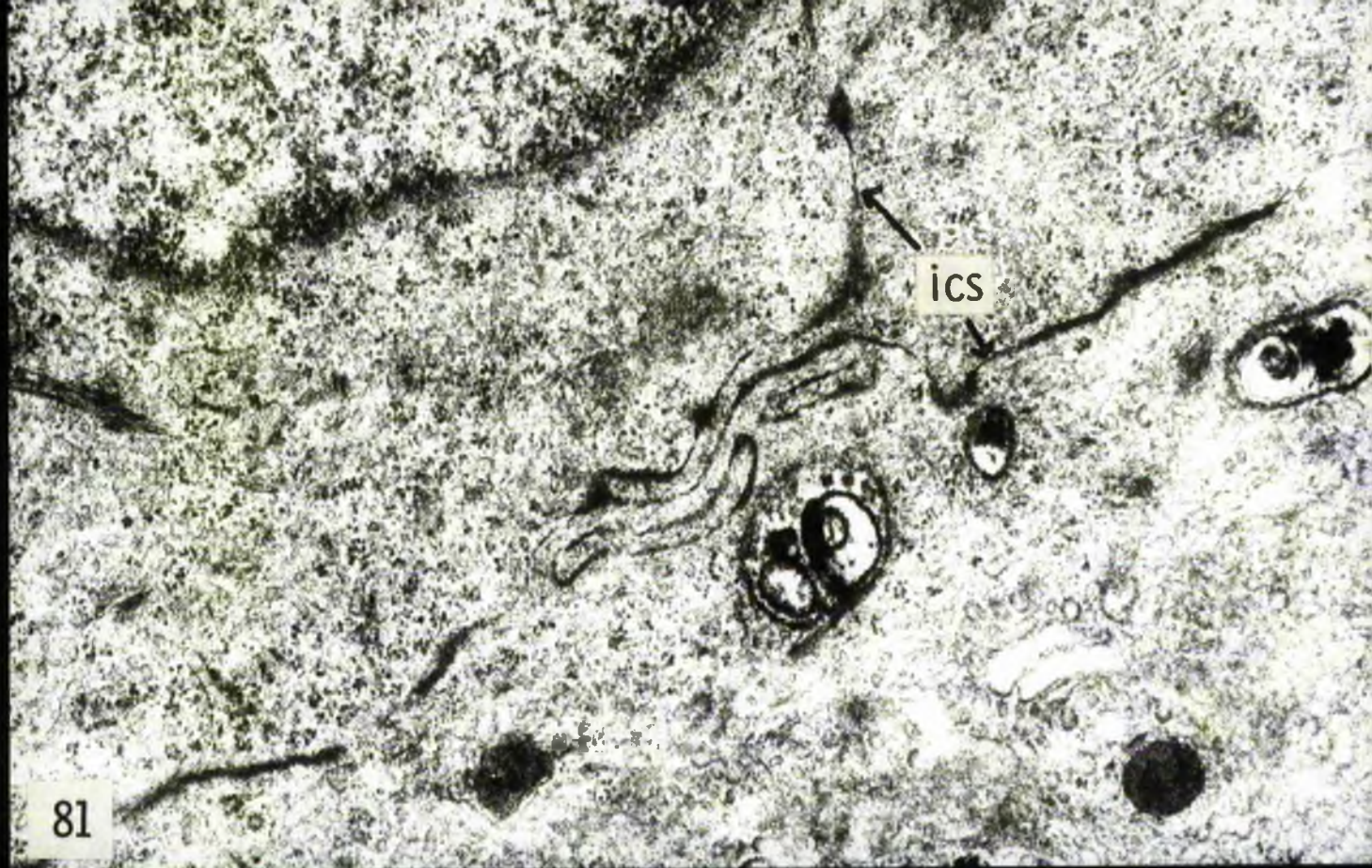


Figure 83: Rat oviductal epithelium in organ culture. Four days culture  
(III-medium+serum).

- a. Note areas of contact (at arrows) between epithelial cells and  
cells of the lamina propria. UA&LC. X6510

SG- secretory granules



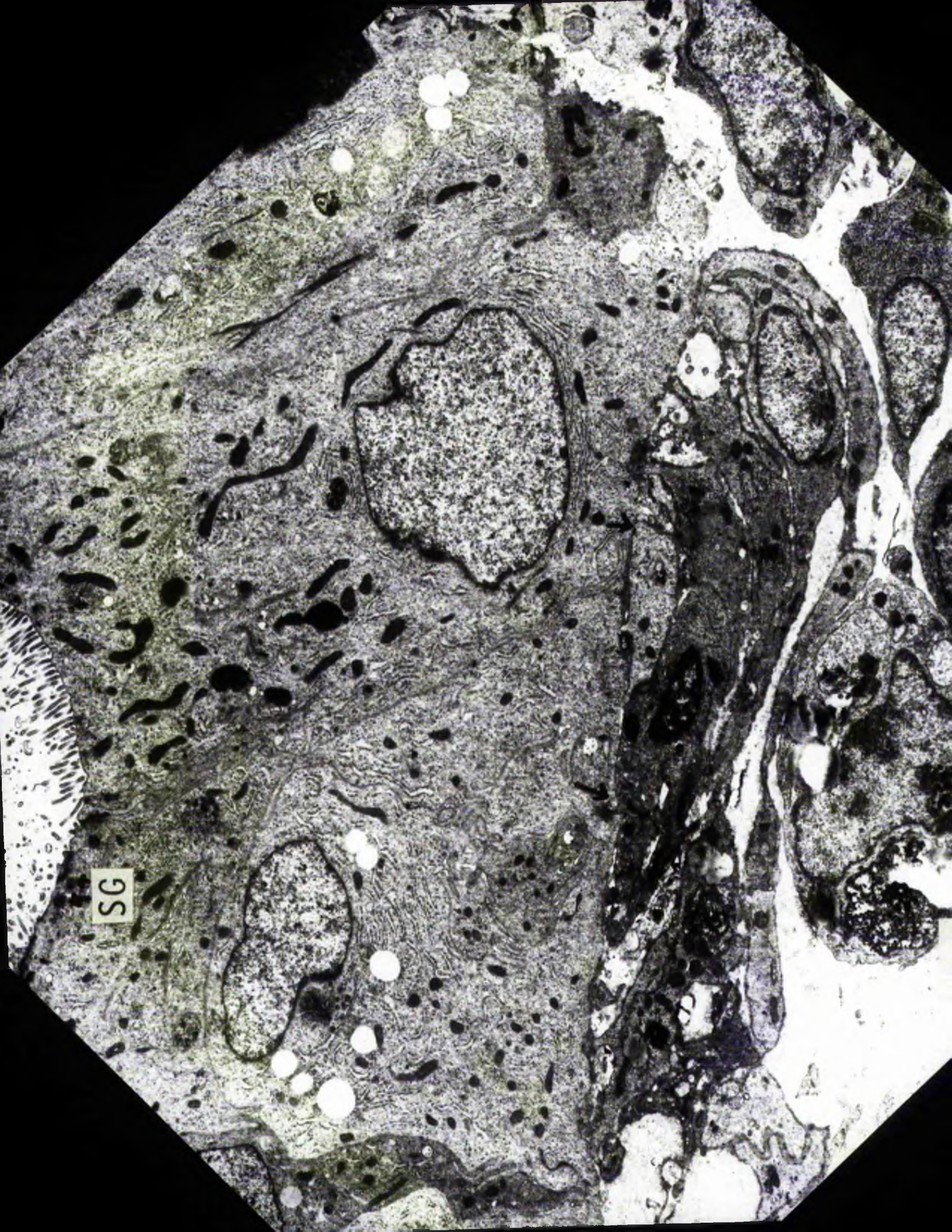
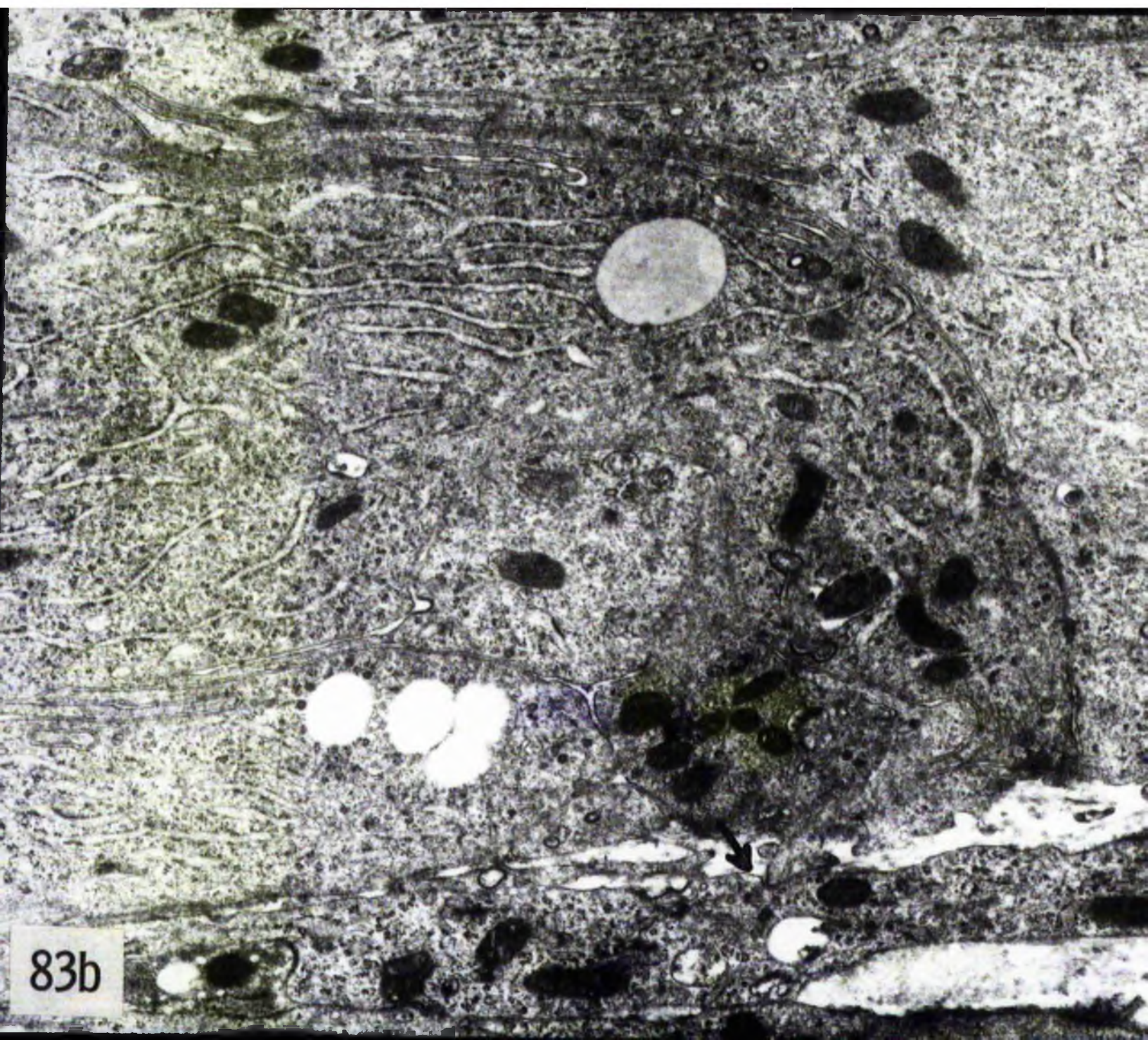




Figure 83(continued):

b. Note absence of basal lamina and pseudopodium  
(at arrow) of epithelial cell extending into lamina  
propria to come in direct contact with a connective  
tissue cell. UA&LC. X23, 670



83b

Figure 84: Rat oviductal epithelium in organ culture. Four days in culture  
(III-medium+serum). UA&LC. X6510

Note thickening of basal lamina in some areas and absence in others.

→ - points of contact between epithelial and connective tissue elements.



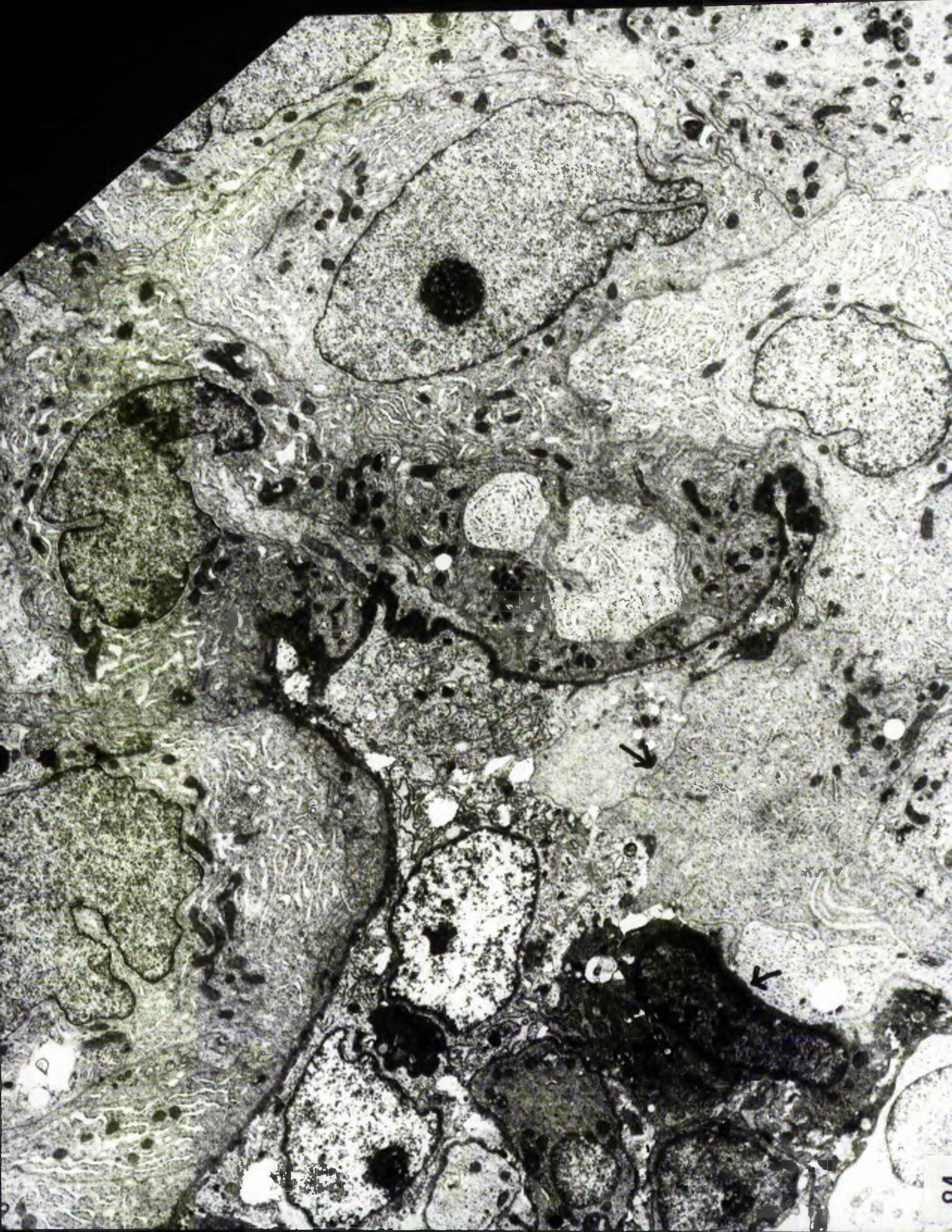


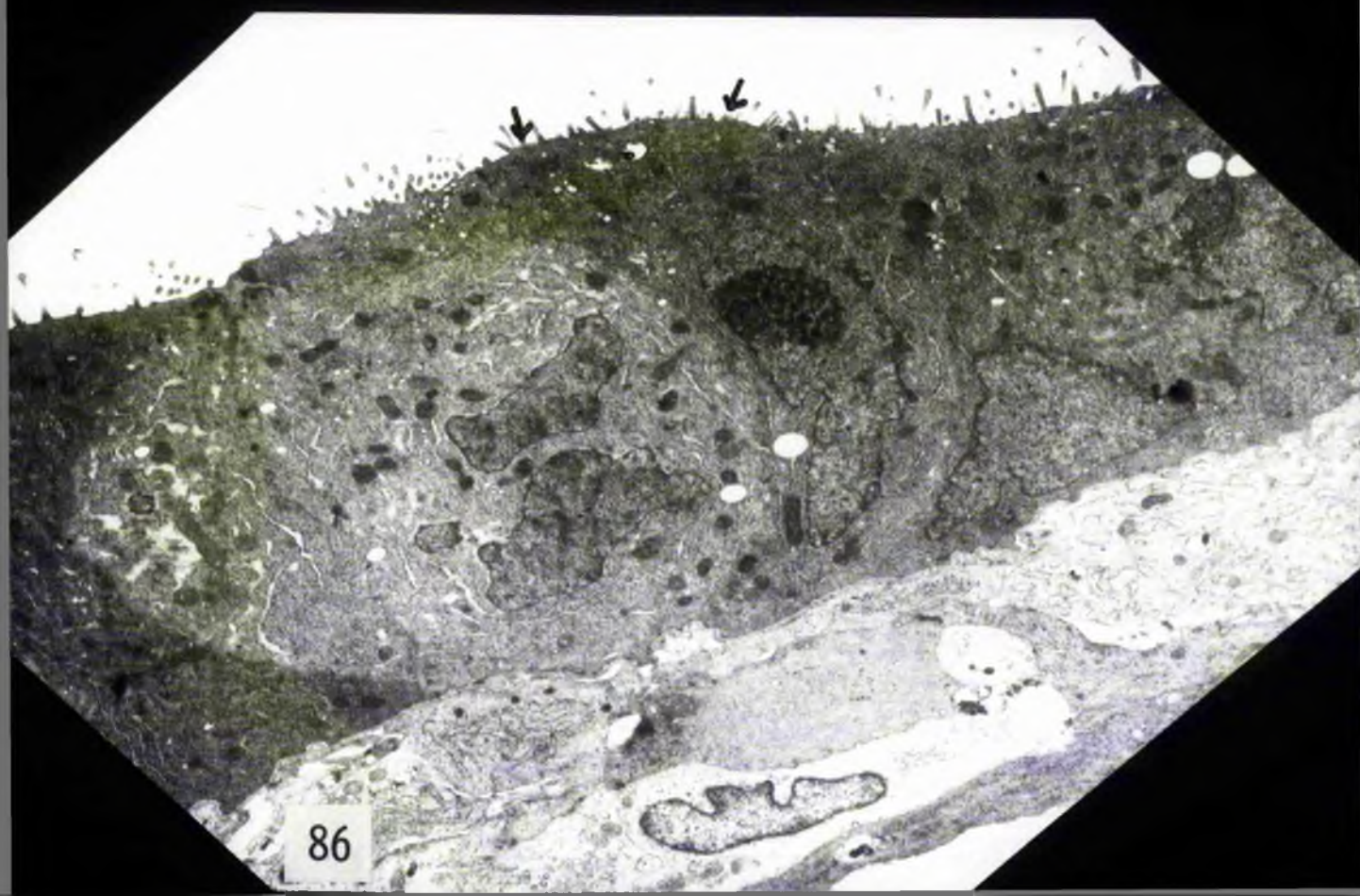
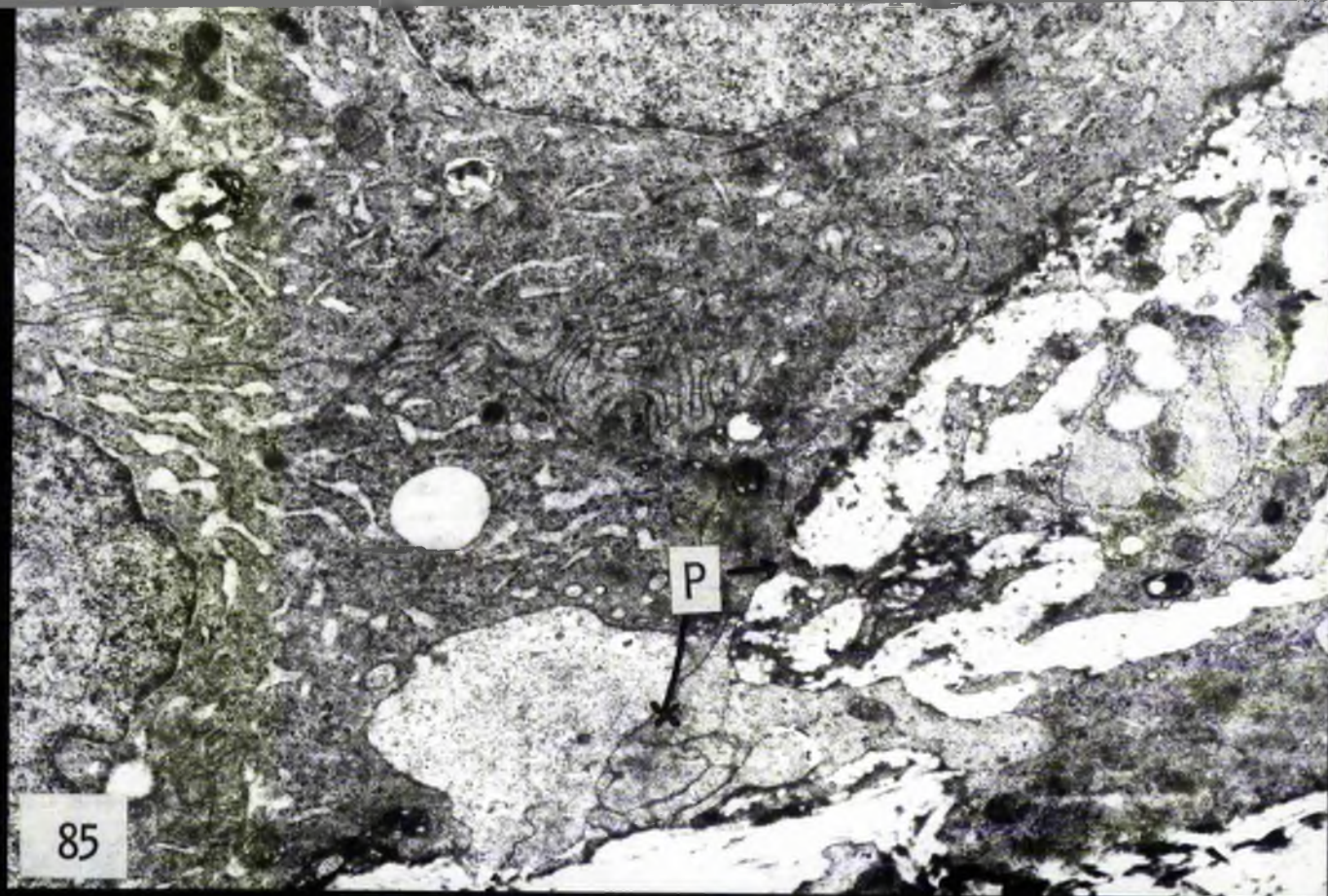


Figure 85: Rat oviductal epithelium in organ culture. Six days  
in culture (III-medium+serum). UA&LC. X23,670

P- pseudopodia from epithelial cells extending into  
lamina propria

Figure 86: Rat oviductal epithelium in organ culture showing  
'dedifferentiation' of the cells. Nine days in  
culture(III-medium+serum). UA&LC. X6510

→ - 'budding' of minute cytoplasmic processes from  
apical cell surface and microvilli





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